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Minireview – Pathogenic Yeasts

Functional analysis of *Candida albicans* Cdr1 through homologous and heterologous expression studies

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Abstract

Candida albicans Cdr1 is a plasma membrane ATP-binding cassette transporter encoded by *CDR1* that was first cloned 30 years ago in *Saccharomyces cerevisiae*. Increased expression of Cdr1 in *C. albicans* clinical isolates results in resistance to azole antifungals due to drug efflux from the cells. Knowledge of Cdr1 structure and function could enable the design of Cdr1 inhibitors that overcome efflux-mediated drug resistance. This article reviews the use of expression systems to study Cdr1. Since the discovery of *CDR1* in 1995, 123 studies have investigated Cdr1 using either heterologous or homologous expression systems. The majority of studies have employed integrative transformation and expression in *S. cerevisiae*. We describe a suite of plasmids with a range of useful protein tags for integrative transformation that enable the creation of tandem-gene arrays stably integrated into the *S. cerevisiae* genome, and a model for Cdr1 transport function. While expression in *S. cerevisiae* generates a strong phenotype and high yields of Cdr1, it is a nonnative environment and may result in altered structure and function. Membrane lipid composition and architecture affects membrane protein function and a focus on homologous expression in *C. albicans* may permit a more accurate understanding of Cdr1 structure and function.

Keywords: *Candida albicans CDR1*; Cdr1p; *Saccharomyces cerevisiae* expression system; ABC protein; N- and C-terminal protein tags; NanoBRET; NanoLuc; HaloTag; pABC3XL; review

Introduction

Fungal infections affect over 1 billion people globally and although many infections are superficial, invasive fungal infections (IFIs) kill more than 1.5 million people each year (Bongomin et al. 2017). The majority of IFIs are caused by *Candida* species and *Candida* blood stream infections have a high attributable mortality of ∼28% (Mazi et al. 2022, Lass-Florl et al. 2024). Although several fungal species can cause disseminated infections, *Candida albicans* is the species that causes invasive candidiasis most frequently (Pfaller et al. 2019). IFIs caused by *C. albicans* are difficult to treat because there are few classes of antifungal drugs available, and the development of drug resistance. Azole antifungals are widely prescribed for fungal infections but *C. albicans* can develop resistance to azoles through a number of mechanisms (Lee et al. 2021). These include overexpression of the drug target Erg11 (also called Cyp51) (Rosana et al. 2015), mutations in Erg11 (Xiang et al. 2013), and overexpression of efflux pumps including the Major Facilitator Superfamily transporter Mdr1 (originally referred to as Ben^r) and ATP-binding cassette (ABC) transporters Cdr1 and Cdr2 (Perea et al. 2001, Cannon et al. 2009).

ABC transporters comprise a very important family of membrane proteins (MPs). They are present in all kingdoms of life and carry out critical cellular functions including nutrient uptake, cell signaling, and export of toxic compounds and xenobiotics. ABC transporters can confer resistance to anticancer drugs (Fletcher et al. 2016) and are responsible for antimicrobial resistance in bacteria (Orelle et al. 2019) and fungi (Cannon et al. 2009). In 1995, a *C. albicans* gene encoding an ABC transporter, Cdr1, was cloned in *Saccharomyces cerevisiae* by Prasad et al. (1995), through its ability to confer resistance to cycloheximide. Expression of Cdr1 was also found to confer resistance to the imidazole miconazole. Subsequent analysis of fluconazole resistance mechanisms in *C. albicans* isolates from HIV/AIDS patients revealed that overexpression of Cdr1 conferred high levels of drug resistance (Lopez-Ribot et al. 1998, Perea et al. 2001). Although *C. albicans* contains six genes (*CDR1*, *CDR2*, *CDR3*, *CDR4*, *CDR11*, and *SNQ2*) in the pleiotropic drug resistance (PDR and ABCG) subfamily of ABC transporters (Lamping et al. 2010), Cdr1 is thought to contribute more than other ABC proteins to fluconazole resistance (Holmes et al. 2008, Tsao et al. 2009).

Due to the role of Cdr1 in the antifungal resistance of clinical *C. albicans* isolates, much research has been focused on developing inhibitors of Cdr1 pump function in order to overcome efflux-mediated resistance and restore fluconazole sensitivity (Schuetzer-Muehlbauer et al. 2003a, Sharma and Prasad 2011, Holmes et al. 2016). Inhibitors of Cdr1 can be sought by screening compounds or by rational structure-based design. Both approaches require the expression of Cdr1. A variety of both heterologous and homologous expression systems have been used to study and purify Cdr1, despite the fact that the membrane environment is a critical determinant of MP function (Neumann et al. 2017, Renard and Byrne 2021). This article reviews the systems

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that have been used to express and study Cdr1, noting their advantages and disadvantages, and identifies future directions for the analysis of Cdr1 structure and function that are also relevant to the study of other important ABC transporters.

Cloning of *C. albicans CDR1* by heterologous expression in *S. cerevisiae*

Candida albicans CDR1 was identified and isolated by screening libraries of *C. albicans* genomic DNA in *S. cerevisiae* JG436 (Mat**a**, *pdr5::Tn5*, *leu2*, *met5*, *ura3-52*, *mak71*, *KRBI*) (Prasad et al. 1995). *Saccharomyces cerevisiae* JG436 has a transposon disruption of *PDR5*—a gene involved in pleiotropic drug resistance (Balzi and Goffeau 1991). Disruption of *PDR5* makes *S. cerevisiae* sensitive to cycloheximide, and other xenobiotics (Leppert et al. 1990, Meyers et al. 1992). Genomic DNA from *C. albicans* 1001 containing the *CDR1* gene was isolated from a centromeric plasmid (pYEURA3) that conferred cycloheximide resistance on *S. cerevisiae* JG436 (Prasad et al. 1995). The open reading frame in the cloned *C. albicans* genomic DNA encoded a protein of 1501 amino acids with a predicted molecular mass of 169.9 kDa. The protein was named '*Candida* drug resistance 1' (Cdr1) and has 56% identity and 73% similarity to *S. cerevisiae* Pdr5 (Prasad et al. 1995).

Fusion of a C-terminal yeast-enhanced green fluorescent protein (GFP) to Cdr1 and expression in *S. cerevisiae* revealed that the protein is predominantly located in the plasma membrane (Lamping et al. 2007). A phylogenetic analysis of the Cdr1 sequence indicated that it aligned with cluster A of fungal PDR ABC transporters, along with *S. cerevisiae* Pdr5 (Lamping et al. 2010). A feature of PDR transporters is that they transport a variety of compounds including xenobiotics and antifungal drugs. Indeed, when Cdr1 was expressed in *S. cerevisiae* it conferred resistance to fluconazole, itraconazole, miconazole, ketoconazole, rhodamine 6G (R6G), cycloheximide, cerulenin, and triton X-100 (Lamping et al. 2007).

Homologous and heterologous expression of Cdr1

In order to investigate the systems that have been used to express and study Cdr1, we conducted a search of research articles indexed in the PubMed database from the discovery of Cdr1 in 1995 to November 2024, using the following keyword combination: *C. albicans* AND (CaCdr1p OR CaCdr1 OR Cdr1p OR Cdr1). It is important to note that some studies related to Cdr1 may not have been retrieved due to the search terms not being present in the title, abstract or keywords of the papers. From an initial retrieval of 490 articles, we removed reviews (these are listed in Table S1), non-English publications, and studies unrelated to *C. albicans* Cdr1, resulting in a pool of 366 articles for further screening (Table S2). Eighteen of these focused solely on the *CDR1* promoter function, 148 investigated the effects of various compounds, growth phases, and environmental factors on native *CDR1* expression levels, 47 examined the drug resistance profiles of clinical isolates overexpressing *CDR1* and *CDR1* deletion strains, and 18 investigated other drug resistance mechanisms (Fig. 1A and Table S2). The remaining 135 studies examined Cdr1 function and structure; 12 papers investigated expression in *C. albicans* clinical isolates and 123 papers utilized laboratory strains to express Cdr1 (Fig. 1A). Over 75% (95) of the 123 articles employed heterologous expression in *S. cerevisiae*, a summary of the species, strains, and expression systems used to express Cdr1 is provided in Table 1 (further details can be found in Tables S3 and S4). Homologous expression in *C. albicans* was utilized in 25 studies (20%), with one study employing insect cells and three, *Escherichia coli*. Integration of the *CDR1* gene into the *S. cerevisiae* (70 studies) or *C. albicans* (23 studies) genomes has been far more widely utilized than plasmid-based systems (25 and 2 studies, respectively) for *CDR1* expression (Fig. 1A). The plasmids used to express Cdr1 are listed in Table 2 (additional details are provided in Table S5). Publication of Cdr1 research peaked in 2004 and has remained at a relatively constant level since 2005 (Fig. 1B).

Suitability of *S. cerevisiae* as an expression host

Saccharomyces cerevisiae has been used in 95 studies to express and investigate *C. albicans* Cdr1 (Fig. 1A). *Saccharomyces cerevisiae* has several advantages for the expression of heterologous proteins, as well as some significant limitations. It is one of the most studied and well-characterized eukaryotic model organisms that is very rarely pathogenic for humans (Aucott et al. 1990). It has both diploid and haploid growth cycles facilitating classical genetic studies as well as gene deletion and manipulation.

An important consideration in heterologous gene expression is the presence of homologous genes in the host organism. Some genes are members of gene families with overlapping activities and functional redundancy. Cdr1 is an example of such a protein. It is an ABC transporter, and *S. cerevisiae* has nine genes that are in the PDR transporter subfamily to which Cdr1 belongs (Lamping et al. 2010). Several of these *S. cerevisiae* PDR transporters can efflux a range of drugs and xenobiotics resulting in a low susceptibility to antifungal drugs that masks the activity of heterologously expressed PDR transporters. This drawback has been overcome by the deletion of several PDR transporter genes in *S. cerevisiae*. Many studies of Cdr1 have been carried out in derivatives of *S. cerevisiae* AD12345678 (*MAT a*, *PDR1–3*, *ura3*, *his1*, Δ*yor1::hisG*, Δ*snq2::hisG*, Δ*pdr5::hisG*, Δ*pdr10::hisG*, Δ*pdr11::hisG*, Δ*ycf1::hisG*, Δ*pdr3::hisG*, Δ*pdr15::hisG*) constructed in André Goffeau's laboratory (Decottignies et al. 1998). This strain is deleted in seven ABC transporters, which makes it highly susceptible to oligomycin and cycloheximide, and expression of *PDR5* in the strain increased resistance to cycloheximide 200-fold (Decottignies et al. 1998). AD12345678, also referred to as AD1-8u$^-$, was further modified by completely deleting *URA3* to form ADΔ (Lamping et al. 2007) and deleting *HIS1* in ADΔ to form ADΔΔ (Sagatova et al. 2015), enabling the completely reliable (~100% accurate) construction of 'correct' uracil or histidine positive transformants using *URA3* or *HIS1*, respectively, as auxotrophic selection markers.

In addition to AD1-8u$^-$ and its derivatives, various other *S. cerevisiae* strains have been used to express and study Cdr1 (Table 1 and Table S3). The JG436 strain, initially employed to clone and isolate *C. albicans* Cdr1, was also used for functional and structural analyses of Cdr1 prior to the widespread adoption of AD1-8u$^-$. Kolaczkowski et al. (2009) developed a strain, designated AK100 (*MAT***a**, *ura3-52*, *trp1*Δ*63*, *leu2*Δ*1*, *his3*Δ*200*, *GAL2*$^+$, *PDR1-3*, *pdr5-*Δ*4::rep500*, *snq2-*Δ*1::hisG*, *yor1-1::hisG*), which was used for Cdr1 expression at the *PDR5* locus under the control of the constitutively active *PDR5* promoter due to the hyperactive mutant transcription factor, Pdr1-3. This strain has also been used to screen for novel antifungal agents (Kolaczkowski et al. 2009, Belofsky et al. 2013). Other strains lacking Pdr5 alone, or Pdr5 and Snq2, or a total of eight plasma membrane ABC transporters have also been

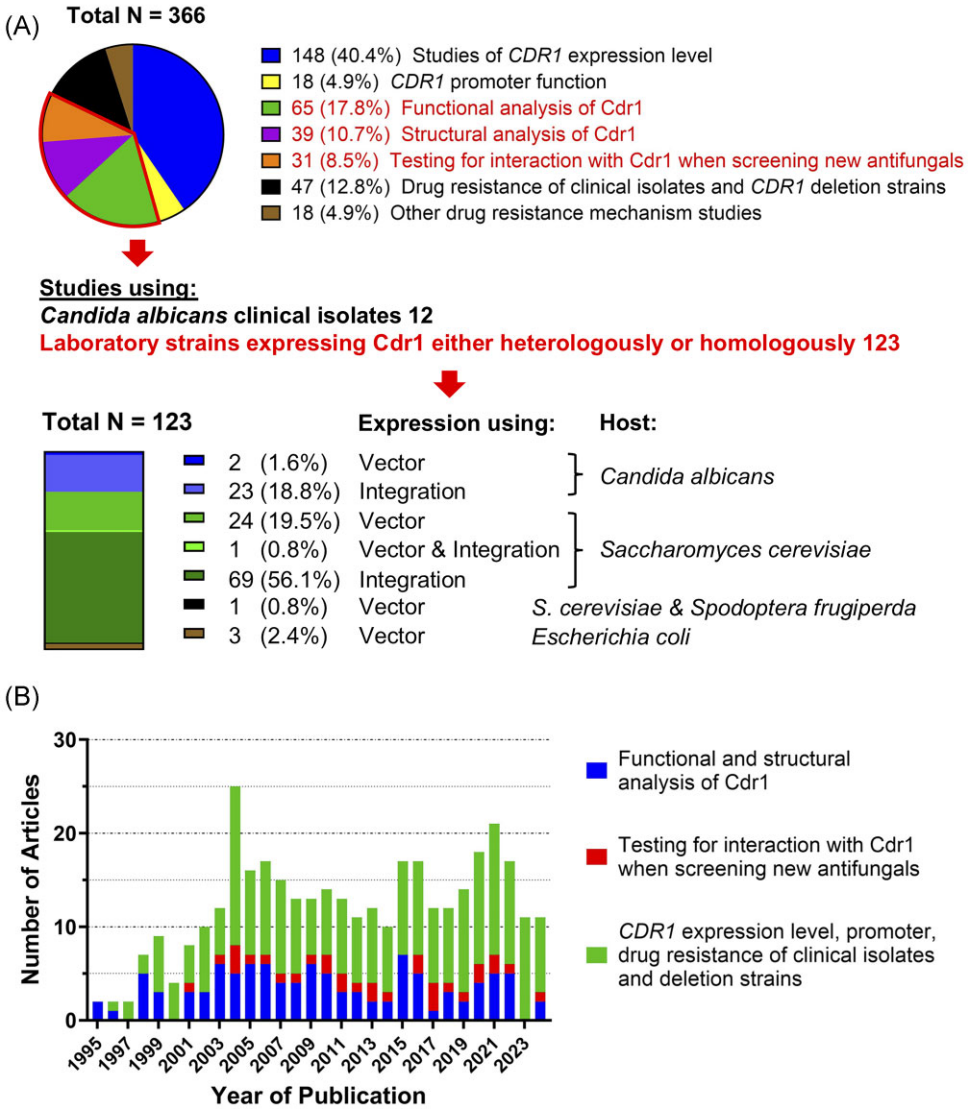


Figure 1. Categorization of research articles related to *C. albicans* Cdr1, indexed in the PubMed database from 1995 to November 2024. (A) A total of 366 studies on Cdr1 were categorized into seven groups based on the main purpose of the study and on the molecular techniques employed to investigate Cdr1 structure and/or function. There were 123 studies that used laboratory strains to study the structure and function of Cdr1 or to screen for novel Cdr1 efflux pump inhibitors. These studies were further subdivided into the types of homologous and heterologous expression technologies used. (B) The number of Cdr1 articles published each year from 1995 to November 2024 categorized into three main groups derived from the seven study categories presented in (A).

used to study Cdr1 (Sanglard et al. 1995, Schuetzer-Muehlbauer et al. 2003a, b). Another strain, TY310, with *PDR1*, *PDR3*, and *PDR5* mutated or knocked out, has been employed to investigate the similarities and differences between *C. albicans* Cdr1 and Cdr2 using the p425GPD plasmid to express *CDR1* (Gauthier et al. 2003). In order to study the effects of membrane fluidity and lipid composition on Cdr1 function, Cdr1 was expressed using the pYEURA3 plasmid in a series of *S. cerevisiae* mutants deleted in either *erg2*, *erg3*, *erg4*, or *erg6* (Smriti et al. 1999). The authors reported that the *erg2*, *erg3*, and *erg6* mutants had increased membrane fluidity, and that all *erg* mutants were more susceptible to cycloheximide, 4-NQO and *o*-phenanthroline when they expressed *CDR1*. Expression of *CDR1* in the *erg* mutants caused a decrease in susceptibility to fluconazole, however, which the authors postulated was due to altered membrane permeability (Smriti et al. 1999). Recently, the first cryo-EM structure of Cdr1 was resolved after expressing the protein in the diploid yeast strain INVSc1, using the p416GAL1 vector (Peng et al. 2024). It is clear that the main approach for heterologous expression and analysis of Cdr1 focuses on reducing endogenous ABC transporter expression while achieving high-level Cdr1 expression.

Expression of Cdr1 in *S. cerevisiae* can be from a plasmid or from a copy of the gene integrated into the genome. Multicopy plasmids can result in high levels of expression of the heterologous gene present on the plasmids, but the expression can vary depending on the plasmid copy number, and due to some of the copies being lost during cell replication. In addition, expression of MPs from multicopy plasmids can result in too much protein being expressed, which can overload the secretory system and prevent proper localization of the MPs. Integrating heterologous genes in the genome ensures a consistent gene copy number and stable inheritance of the gene. Although only present in one copy per haploid cell, protein expression can be controlled with a suitable promoter. Inducible/repressible promoters used to express episomal

Table 1. Species, strains, and transformation methods used to express Cdr1.

Expression host	Strain	Vector/integration	Number of studies	References (PMID[a])
C. albicans	DSY1050	pYM70 (*ACT1* promoter), pYM71 (*GAL1* promoter)	1	20348384
	CAI-4/ΔCDR1	pRC2312 (*HEX1* promoter)	2	15486081, 22205973
	CAF2-1, CAF4-2, CAI-4	Integration (*CDR1* locus)	12	11967831 and[b]
	SC5314	Integration (*CDR1* locus)	1	23979757
	DSY3040, DSY3041	Integration (*CDR1* locus)	1	20818920
	GU4, GU5, DSY294, DSY296	Integration (*CDR1* locus)	1	18268086
	DSY4684	Integration (*CDR1* locus allele A)	1	26973635
	STY31	Integration (*CDR1* locus allele A)	1	27402010
	DSY449, DSY653, DSY465	Integration (*CDR1* locus)	2	22205973, 33669913
	RM1000	Integration (*CDR1* locus)	1	9605502
	CAI-4/ΔCDR1	Integration (*RP10* locus; *ACT1* promoter)	2	11967831, 25009090
	SC5314	Integration (*RPS1* locus; Tet promoter)	1	20700541
	SN152	Integration (*LEU2* locus one allele; *ADH1* promoter/*CDR1* other allele)	1	36618949
S. cerevisiae	TY310	p425GPD (*GPD* promoter)	1	12709320
	DSY566, YKKB-13, YMM-ABC8, YYM4	YEp24 (pDS243, native promoter)	7	8585712 and[c]
	JG436	pYEURA3 (native promoter)	6	7614555 and[d]
	BY4741	pYEURA3 (native promoter)	1	20608983
	AD1234568	pYEURA3 (native promoter)	1	14665469
	AD1-8u$^-$	pYEURA3 (native promoter)	8	9453158 and[e]
	ABC287, ABC271, ABC261, ABC283, ABC265 (a series of *erg* mutants)	pYEURA3 (native promoter)	2	10227177, 12435664
	BY4741 ΔPdr5, INVSc1	p416GAL1 (*GAL1* promoter)	1	39242571
	SY1	pVT101-U (*ADH1* promoter)	1	9580031
	AD1-8u$^-$	Integration (*PDR5* locus; *PDR5* promoter)	58	11709310 and[f]
	ADΔ	Integration (*PDR5* locus; *PDR5* promoter)	5	16185821 and[g]
	ADΔΔ	Integration (*PDR5* locus; *PDR5* promoter)	2	33568458, 34180894
	AK100	Integration (*PDR5* locus; *PDR5* promoter)	5	19188399 and[h]
	YEM139	Not mentioned	2	15380213, 15380214
Spodoptera frugiperda	Sf9	pBacPAK8 (AcMNPV polyhedrin promoter)	1	9605504
E. coli	BL21(DE)pLysS	pGex2T (Tac promoter)	3	12962507 and[i]

[a]PubMed identifier.
[b]15 845783, 16 048959, 16246085, 26696990, 28602766, 31546699, 31826306, 33669913, 33753842, 34069257, and 36077373.
[c]10052898, 12657057, 13678837, 14695809, 16643022, and 26482310.
[d]9453158, 9605504, 10029989, 11120970, and 12054050.
[e]10029989, 11870854, 14665469, 16782311, 17305773, 18794383, and 26300857.
[f]14665469, 15047528, 15105135, 15190023, 15325261, 15381123, 15850398, 15937063, 16475832, 16569823, 16622073, 16942600, 17126815, 17144665, 17513564, 17924650, 17967417, 18056285, 18710914, 19393219, 19470507, 19577533, 19615977, 20546701, 20739103, 21768514, 21895791, 22166216, 22203607, 22788839, 24628911, 25437914, 26048893, 26053667, 26349561, 27145238, 27251950, 27431223, 27569110, 27821447, 28106996, 28479022, 28559186, 29410026, 30043631, 30157240, 30348662, 30639597, 31093957, 31734312, 32344123, 33394279, 34780272, 35496110, 35773582, 36224581, and 38959728.
[g]17513564, 34180894, 38158037, and 38813525.
[h]19460101, 19549107, 23122779, and 23631483.
[i]14550284 and 18498759.

or integrated *CDR1* include the promoters of the *C. albicans GAL1* and *HEX1* genes (Niimi et al. 2004, Basso et al. 2010), the promoters of the *S. cerevisiae GAL1* and *PDR5* genes (Peng et al. 2024), and the Tet and tac promoter from *E. coli* (Jha et al. 2003, Lettner et al. 2010) (Table 2). Constitutive promoters have also been used, namely the *C. albicans ACT1* and *S. cerevisiae GPD* promoters (Gauthier et al. 2003, Basso et al. 2010), the *ADH1* promoters from *C. albicans* and *S. cerevisiae*, and the polyhedrin promoter from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Krishnamurthy et al. 1998) (Table 2 and Table S5).

The *S. cerevisiae PDR5* promoter has been widely used in strains such as AD1-8u$^-$ to express Cdr1 (Table 1). *Saccharomyces cerevisiae* AD1-8u$^-$ and its derivatives contain *PDR1-3*, a form of *PDR1* with a gain-of-function mutation that increases expression of genes containing pleiotropic drug response element motifs in their promoters (Katzmann et al. 1996). Transcriptional analysis has shown

Table 2. Plasmids and promoters used to express Cdr1.

Plasmid	Promoter used for *CDR1* expression	GenBank	ATCC	Description	References (PMID)
p425GPD	Sc[a]*GPD* (glyceraldehyde-3-phosphate dehydrogenase)		87359	High copy episomal *S. cerevisiae* and *E. coli* shuttle and *S. cerevisiae* expression vector	12709320
YEp24	Native (Ca[b]*CDR1*)	L09156.1	37051	High copy episomal *S. cerevisiae* and *E. coli* shuttle vector	8585712 and[c]
pYM70	*CaACT1* (actin)	GU937092.1		High copy episomal *S. cerevisiae* and *E. coli* shuttle and yeast expression vector	20348384
pYM71	*CaGAL1* (galactokinase)			High copy episomal *S. cerevisiae* and *E. coli* shuttle and yeast expression vector	20348384
pBacPAK8	AcMNPV polyhedrin promoter	U02446		High copy episomal *E. coli* cloning vector for transfection of and expression in Sf9 insect cells	9605504
pGex2T	Tac			High copy episomal *E. coli* vector for expressing GST fusion proteins	12962507 and[d]
pYEURA3	Native (*CaCDR1*)	U02457.1		Low copy episomal centromeric *S. cerevisiae* and *E. coli* shuttle vector	7614555 and[e]
pRC2312	*CaHEX1* (hexosaminidase)		77411	High copy episomal *S. cerevisiae*, *C. albicans* and *E. coli* shuttle vector	15486081
p416GAL1	*ScGAL1* (galactokinase)		87332	Low copy episomal *S. cerevisiae* and *E. coli* shuttle and *S. cerevisiae* expression vector	39242571
pVT101-U	*ScADH1* (alcohol dehydrogenase)			High copy episomal *S. cerevisiae* and *E. coli* shuttle vector	9580031

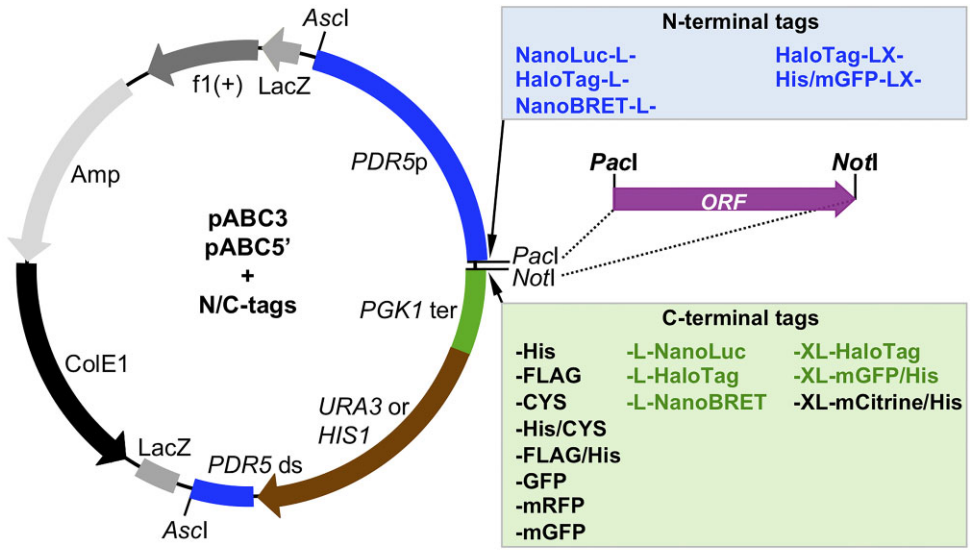
[a]*Saccharomyces cerevisiae*.
[b]*Candida albicans*.
[c]10052898, 12657057, 13678837, 14695809, 16643022, and 26482310.
[d]14550284 and 18498759.
[e]9453158, 9605504, 10029989, 10227177, 11120970, 11870854, 12054050, 12435664, 14665469, 16782311, 17305773, 18794383, 20608983, and 26300857.

that the *pdr1-3* mutation results in the induction of a range of genes including those involved in sphingolipid biosynthesis as well as ABC transporters (DeRisi et al. 2000, Kolaczkowski et al. 2004). The pleiotropic network of genes upregulated by Pdr1-3 may explain the high level of Cdr1 expression in the plasma membrane of *S. cerevisiae* cells containing *CDR1* integrated under the control of the *PDR5* promoter (Lamping et al. 2007). It is noteworthy that AD1-8u$^-$, ADΔ, and ADΔΔ are all deleted in *PDR3*, which is a homologue of *PDR1* and which also controls genes involved in pleiotropic drug resistance (Delaveau et al. 1994). Another way of controlling the expression of Cdr1 integrated in *S. cerevisiae* at the *PDR5* locus is by introducing hairpins in the 5′ untranslated region just upstream of the AUG start codon (Lamping et al. 2013). By inserting hairpins with different lengths of GC-paired stems or sizes of loops, the hyperexpression of Cdr1 in *S. cerevisiae* ADΔ could be predictably downregulated up to 125-fold by blocking AUG-start codon scanning of the ribosomal 43S preinitiation complex. Every additional GC-pair in the GC-rich stem loop upstream of the AUG start codon caused an additional 2.8-fold reduction in Cdr1 expression with an equally strongly reduced resistance to the Cdr1 efflux pump substrate, fluconazole. This study confirmed that despite the exceptionally high expression level, all Cdr1 molecules are functional and contribute to the fluconazole resistance of cells overexpressing Cdr1 (Lamping et al. 2013).

A disadvantage of using *S. cerevisiae* as a heterologous host for the expression of *C. albicans* proteins is that *S. cerevisiae*, unlike *C. albicans*, is not a CTG-clade yeast. CTG-clade fungal species, including *C. albicans*, translate CUG codons as serine instead of leucine (Santos and Tuite 1995). This difference can lead to structural or functional changes when *C. albicans* proteins are expressed in *S. cerevisiae*. *Candida albicans CDR1*, however, does not contain any CTG codons, and so its expression in *S. cerevisiae* is unaffected in this respect. There are other potential drawbacks of heterologous expression of Cdr1 in *S. cerevisiae*, as with other heterologous expression systems, that include non-native posttranslational modification, and differences in membrane composition and thickness (Kaur and Bachhawat 1999, Pasrija et al. 2008).

A suite of plasmids and protein tags for biological assays and purification of Cdr1

There is a vast array of protein tags available for myriads of potential biological or medical applications (Vandemoortele et al. 2019). Over the past two decades, we have created a number of pABC3 (containing the *URA3* selection marker) and pABC5′ (containing the *HIS1* selection marker) (Lamping et al. 2007) derivative plasmids that direct the stable integration of a heterologous expression cassette at the chromosomal *PDR5* locus of the *S. cerevisiae* ADΔΔ host (Fig. 2). All these plasmids are used in the same fashion by: (i) cloning *CDR1* or any other ORF into the unique and rare 8 bp *PacI*/*NotI* restriction enzyme cutting sites; (ii) digesting the resulting plasmids with *AscI*; and (iii) transforming



Sequences of N-terminal linkers		
DNA	**=**	**amino acid**
-*Pac*I + A-	=	-LIK-
-L + *Pac*I + A-	=	-GSGGS-LIK-
-LX + *Pac*I + A-	=	-GGSGG-LEVLFQ/GP-LIK-

Sequences of C-terminal linkers	
DNA	**= amino acid**
-G + *Not*I -	= -GGR-
-G + *Not*I + L-	= -GGR-GSGGS-
-G + *Not*I + XL-	= -GGR-LEVLFQ/GP-GSGGS-

NanoBRET = NanoLuc-GSSGGS-HaloTag

mCitrine = mGFP + F64L-V68L-Q69M-T203Y

Figure 2. A suite of plasmids for the overexpression of heterologous ORFs such as Cdr1 with a versatile range of N- and C-terminal tags. All plasmids are derivatives of pABC3 and pABC5′ (Lamping et al. 2007) and can be used by ligating the ORF of interest (eg *CDR1*) into the unique 8-bp *Pac*I and *Not*I restriction enzyme sites. To ensure in-frame fusions of the N- (upper box) and C-terminal (lower box) tags with the ORF, an additional nucleotide (bold) needs to be attached to the '*Pac*I' (TTAATTAA**A**) and '*Not*I' (**G**GCGGCCGC) primers for PCR amplification of the desired ORF. All N- and C-terminal tags are available upon request as pABC3 and pABC5′ derivatives containing either the *URA3* or the *HIS1* auxotrophic selection marker, respectively. The amino acid sequences introduced between the N- and C-terminal tags and the ORF are listed in the left and right tables at the bottom of the Figure, respectively: L = linker, X = HRV 3C protease cleavage site for the removal of N- or C-terminal tags. See text for further details.

the ADΔΔ host with the linear *Asc*I transformation cassette and selecting for uracil prototrophic transformants that have the entire transformation cassette stably integrated at the chromosomal *PDR5* locus via two homologous cross-over events (Fig. 3C). The original pABC3 and pABC5′ derivative plasmids only offered C-terminal affinity or fluorescence tags that were separated from the tagged protein by a short GGR-linker (Fig. 2). None of the C-terminal tags affected the expression or function of Cdr1 in the ADΔ host (Lamping et al. 2007). Tag options included hexahistidine (His), tetracysteine (Cys) (Griffin et al. 1998, Thorn et al. 2000) or double FLAG/His (Kaneko et al. 2004) or His/Cys affinity tags for the purification and chemical- or immuno-detection of the protein of interest, and a yeast enhanced green fluorescence protein yEGFP3 (GFP) (Cormack et al. 1997) or a monomeric red fluorescent protein mRFP1 (mRFP) tag (Campbell et al. 2002) for the visualization and study of one or two heterologous proteins coexpressed in the same ADΔ host (Lamping et al. 2005).

Since then, the pABC3 and pABC5′ transformation cassettes have been significantly improved. The extension of the GGR-linker with a five amino acid GSGGS-linker (L; Fig. 2) was necessary to prevent the inactivation of the *Candida utilis* Cdr1 transporter by the C-terminal GFP tag (Watanasrisin et al. 2016). Further improvements saw the creation of a versatile C-terminal mGFP/His double tag with an eight amino acid HRV 3C protease cleavage site (X) between the tag and the ORF to enable the removal of the mGFPHis tag (Fig. 2) (Madani et al. 2021). The monomeric GFP-A206K (mGFP) variant prevents potential GFP-dimerization at high protein concentrations (von Stetten et al. 2012), and the additional three amino acid GGS-linker between mGFP and the His tag ensures proper surface exposure of the His tag to maximize the binding efficiency of the tagged protein to nickel affinity purification resins. N-terminal tag versions of these plasmids were also created. The N-terminal mGFPHis double tag is, however, a mirror image (i.e. HismGFP) of the C-terminal mGFPHis double tag (Fig. 2). A C-terminal mCitrineHis double tag in pABC3 has also been generated. mCitrine is a much improved version of yellow fluorescent protein (Griesbeck et al. 2001, Sheff and Thorn 2004) and offers an additional fluorophore for multicolor fluorescence tagging and protein–protein interaction (PPI) studies. These novel N- or C-terminal mGFPHis double tags did not alter the plasma membrane localization nor the efflux pump function of Cdr1 or Pdr5 (Fig. 3B). In addition, in-gel fluorescence of the mGFP tag (Madani et al. 2021) enables the efficient and reliable quantification of protein expression levels (Fig. 3B).

The bioluminescence resonance electron transfer (BRET) technology is a widely used technique to study PPIs. Its general applicability, however, is diminished by a limited sensitivity and small dynamic range (Machleidt et al. 2015). The NanoBRET technology has addressed these issues by combining an extremely bright luciferase NanoLuc (Hall et al. 2012) with a long-wavelength fluorophore, HaloTag (Los et al. 2008, Machleidt et al. 2015), that ensures almost complete separation of the donor and acceptor spectra. NanoLuc, a luciferase from *Oplophorus gracilirostris* is ~100 times brighter than the commonly used firefly or *Renilla* luciferases (Hall et al. 2012). NanoLuc is an ideal luciferase for biomedical research for several reasons. It is very small (~19 kDa),

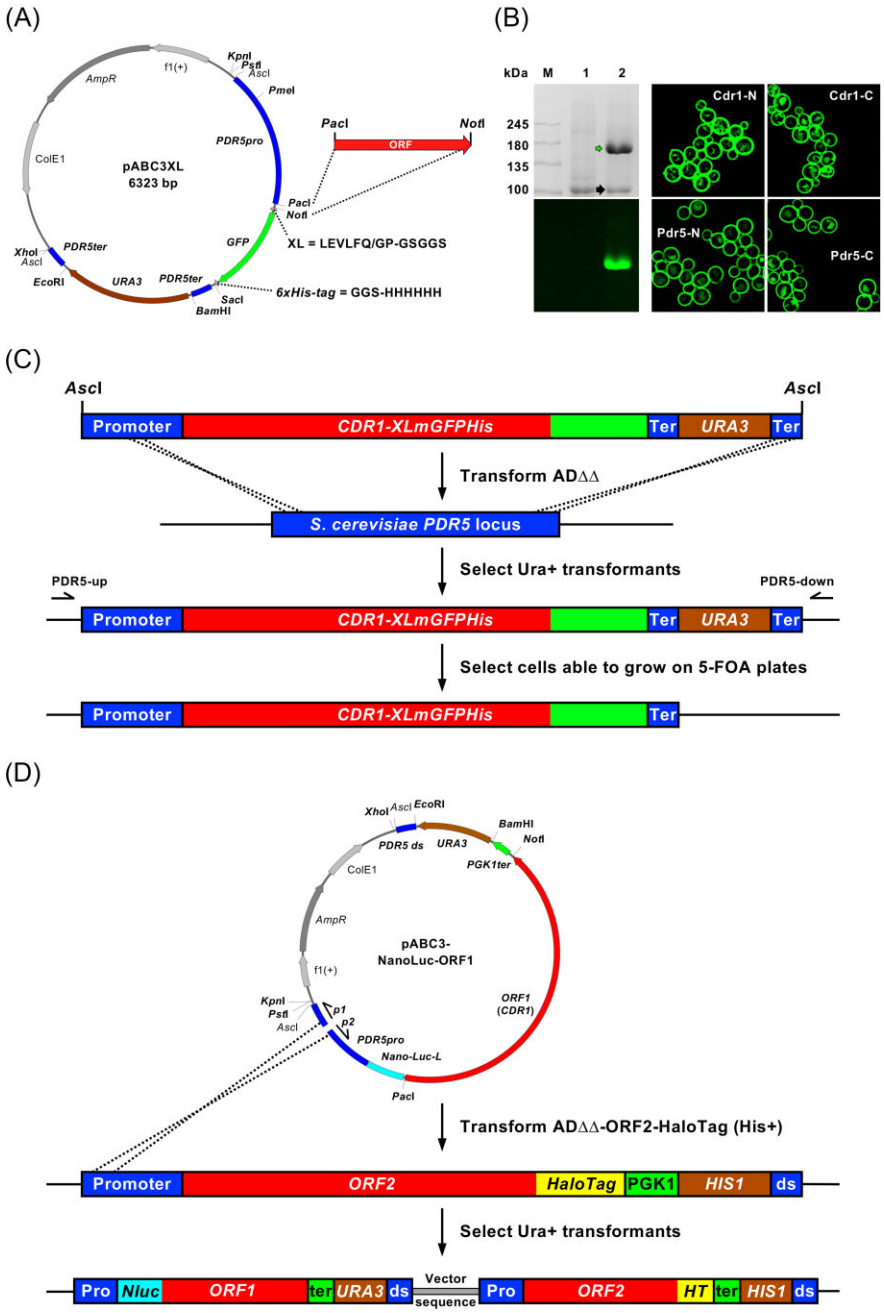


Figure 3. Graphical illustration of pABC3XL and the creation of tandem-gene arrays in the *S. cerevisiae* ADΔΔ genome for the overexpression of *CDR1* and/or *PDR5* with N- and C-terminal mGFPHis double tags. (A) Map of pABC3XL derived from pABC3-XLmGFPHis (James et al. 2021). Individual DNA elements within the *AscI* transformation cassette of pABC3XL can be excised and easily replaced using the unique restriction enzyme cutting sites (bold) between each element. pABC3XL also has a 'recyclable' *URA3* 'blaster cassette' between the 196 bp *PDR5* terminator repeats (*PDR5ter*) and a unique 8-bp *PmeI* site for the directed integration of *PmeI*-digested linear ORF-containing plasmids into the genomic *PDR5* promoter region of *S. cerevisiae* ADΔΔ cells [see (D) below and text for further details]. (B) SDS-PAGE analysis and in-gel fluorescence of 20 µg plasma membrane proteins of *S. cerevisiae* ADΔΔ cells transformed with empty pABC3 cassette (lane 1) or the pABC3-Cdr1-XLmGFPHis cassette (lane 2) showing high levels of Cdr1 expression (upper arrow). The lower arrow indicates Pma1. To the right are confocal microscopy images of *S. cerevisiae* ADΔΔ cells expressing *C. albicans* Cdr1 (top) or *S. cerevisiae* Pdr5 (bottom) each with either an N-terminal (left) or C-terminal (right) mGFPHis double tag. (C) Cloning strategy for the construction of *S. cerevisiae* ADΔΔ cells overexpressing *CDR1*-mGFPHis. Transformation with the linear *AscI*-digested and agarose gel-purified transformation cassette and the selection of uracil prototrophic transformants leads to the generation of ADΔΔ-CaCDR1-mGFPHis (top three lines). Correct transformants are verified by PCR amplification with primers PDR5-up and PDR5-down and sequencing the entire ORF. Cells able to grow on 5-FOA plates have lost the *URA3* marker through recombination of the repeated *PDR5* terminator (Ter) and retain the heterologous ORF, in this case *CDR1*-mGFPHis (bottom line). These cells can be transformed again with another DNA cassette using the same *URA3* auxotrophic selection marker for transformation. (D) Construction of a tandem array of two ORFs, ORF1 with an N-terminal NanoLuc and ORF2 with a C-terminal HaloTag, stably integrated at the genomic *PDR5* locus with both ORFs regulated by their respective *PDR5* promoters (Pro). This strain was generated by two successive transformations of the ADΔΔ host using *HIS1* and *URA3* as auxotrophic selection markers. In the first step, the ADΔΔ host was transformed with the *AscI*-digested and *HIS1*-containing cassette of pABC5′-ORF2-HaloTag resulting in integration at the genomic *PDR5* locus (top line). In the second step, this strain was transformed with the linear plasmid pABC3-NanoLuc-ORF1 using *URA3* to select for Ura$^+$ transformants that had the two ORFs stably integrated in tandem at the genomic *PDR5* locus of the ADΔΔ host (bottom line). Integration of pABC3-NanoLuc-ORF1 into the genomic *PDR5* promoter of ADΔΔ-ORF2-HaloTag was achieved at high frequency by linearizing the plasmid by PCR before transformation (see text for further details).

has no posttranslational modification, no apparent subcellular partitioning bias, it is very stable up to 55°C, active over a broad pH range (pH 5–9), a monomer with no propensity for oligomerization, it has no disulfide bonds and, unlike GFP tags, it can also be used to tag extracellular domains, and is used extensively to study N-terminally tagged G-protein coupled receptor proteins (Hall et al. 2012).

HaloTag is a 33-kDa mutant bacterial haloalkane dehalogenase from *Rhodococcus rhodochrous* (Los et al. 2008) that covalently binds alkyl–enzyme intermediates at aspartic acid D106. The haloalkane ligand can be a small fluorescent ligand, a solid phase chromatography resin, or any other kind of reactive ligand. The HaloTag provides a very versatile system for many biomedical applications (England et al. 2015). The HaloTag can be used as a fluorescent tag for *in vivo* and *in vitro* studies of protein localization, protein function, ligand-binding, and PPIs. However, it can also be used for one-step purification of large protein complexes or the purification of proteins of interest for 2D or 3D crystallography without the need for multiple purification steps as is usually the case when using other affinity purification tags (Kaneko et al. 2004).

The creation of pABC3 and pABC5′ derivatives enables the tagging of the N- or C-termini of proteins with NanoLuc, HaloTag, or NanoBRET [the 'positive PPI control' tag with the bioluminescent donor (NanoLuc) attached to the HaloTag fluorophore acceptor by a six amino acid GSSGGS-linker], separated by an eight amino acid linker from the protein of interest (Fig. 2). The plasmids also allow the option to release the protein of interest from its HaloTag extension that is covalently bound to the solid phase HaloTag chromatography resin by HRV 3C protease cleavage. Preliminary experiments have confirmed that none of these N- or C-terminal NanoBRET tags alter the expression or function of Cdr1, and both NanoLuc and HaloTag, including the positive NanoBRET control tag, all of which were developed for PPI studies in mammalian cells, can also be used for PPI studies in *S. cerevisiae* ADΔΔ. Although we have not yet detected any positive PPIs in *S. cerevisiae*, we are currently using the NanoBRET technology to investigate whether Cdr1 forms functional homodimers or multimers in native yeast membranes. Plasmid pABC3XL (Fig. 3A) was designed to enable PPI studies as illustrated in Fig. 3(C) and (D). pABC3XL has unique restriction enzyme cutting sites placed between each individual DNA element within the *Asc*I transformation cassette (Fig. 3A) that enable the excision and replacement of any of these elements. In PPI studies, two proteins of interest can be expressed at equimolar amounts from their respective *PDR5* promoters of tandem-ORF arrays integrated at the genomic *PDR5* locus, as indicated in Fig. 3(D). This is achieved by transforming the His^+ *S. cerevisiae* ADΔΔ strain containing ORF2 with, for instance, a C-terminal HaloTag stably integrated at the genomic *PDR5* locus (i.e. ADΔΔ-ORF2-HaloTag created with pABC5′-ORF2-HaloTag; top line Fig. 3D) with a second ORF-containing pABC3 derivative plasmid that has been linearized at the *PDR5* promoter by polymerase chain reaction (PCR) amplifying the entire pABC3-NanoLuc-ORF1 plasmid with primers p1 and p2 (Fig. 3D) and selecting for Ura^+ transformants. Linearizing plasmid pABC3-NanoLuc-ORF1 at the *PDR5* promoter ensures that the second ORF-containing plasmid integrates at high frequency via a single homologous recombination event into the genomic *PDR5* promoter region just upstream of the first ORF. Future pABC3 derivative plasmids containing the various N- and C-terminal tags, a unique *PmeI* restriction site in the *PDR5* promoter and a recyclable *URA3* blaster cassette will improve the creation of tandem gene arrays stably integrated at the genomic *PDR5* locus. This will negate the need for multiple selection markers or for the PCR amplification of linear plasmids. Such tandem arrays of two genes of interest expressed at equimolar amounts have many additional applications apart from the PPI studies mentioned above. All the plasmids presented in Figs 2 and 3 are available upon request.

Other heterologous expression systems

For structural and functional analysis of MPs using X-ray crystallography or cryo-EM, it is essential to produce sufficient quantities of the MP, ensure proper folding within the membrane, and accurate posttranslational modifications (such as glycosylation and phosphorylation) that affect functionality and stability (Evans et al. 1995). Several heterologous expression systems, other than *S. cerevisiae* have been used to study Cdr1.

Escherichia coli

Escherichia coli expression systems offer several advantages, including rapid growth, cost-effective nutrient requirements, a quick and simple transformation process, and inducible protein expression. *E. coli*, however, also has notable limitations. Importantly, the *E. coli* membrane composition is different to those for human and yeast cells, including the absence of sterols. Often, expressed proteins accumulate in inclusion bodies, there can be challenges in maintaining the expression plasmid due to the toxicity of the heterologous protein, and there can be degradation of the expressed protein by bacterial proteases. In addition, differences in codon usage between prokaryotic *E. coli* and eukaryotic cells can affect the expression and folding of fungal MPs (Evans et al. 1995).

Prasad's group cloned a 512-amino acid fragment of Cdr1 containing the N-terminal nucleotide-binding domain (NBD), NBD512, as a GST fusion protein in *E. coli* BL21(DE3)pLysS (Jha et al. 2003). Expression of NBD512 was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), the cells were lysed, and the 58-kDa NBD512 protein fraction was affinity purified. Due to its moderate solubility, NBD512 was easily purified and accounted for ∼25% of the total cellular protein. NBD512 exhibited ATPase activity with a *Km* of 0.8–1.0 mM and a *Vmax* of 147–160 nmol/min/mg protein. This demonstrated that the NBD1 of Cdr1 when expressed in *E. coli* has ATPase activity (Jha et al. 2003) although it is important to note that in its native state adenosine triphosphate (ATP) hydrolysis by Cdr1 occurs in a composite nucleotide-binding site (CNBS) containing elements of NBD1 and NBD2 (Prasad et al. 2019). Two further studies from the same laboratory investigated the nucleotide binding and catalytic mechanism of ATP hydrolysis by expressing mutated versions of NBD1 in *E. coli* BL21(DE3)pLysS, purifying the proteins, measuring their ATPase activity and undertaking fluorescence resonance energy transfer analysis (Rai et al. 2005, 2008). To our knowledge, the entire Cdr1 protein has not been expressed in *E. coli*.

Baculovirus/insect cell lines

The baculovirus/insect cell system offers significant advantages for the expression of heterologous proteins, including the ability to perform posttranslational modifications characteristic of eukaryotic proteins and to express large heterologous proteins in correctly folded forms. When insect cells (Sf9) are infected with a baculovirus containing the target gene under the control of the strong late polyhedrin promoter, the amount of heterologous protein produced by Sf9 cells accounts for ∼3% of the total membrane vesicle proteins (Tsai et al. 2020). This system has been reported

to enable large-scale production of heterologous proteins from infected Sf9 cells while preserving the native multimeric structure of the proteins, making it suitable for purification, reconstitution, and structural studies (Tsai et al. 2020).

In early studies by Prasad's group, a truncated version of *CDR1*, referred to as Δ*CDR1*, was generated by deleting 79 amino acids from the C-terminus, resulting in the removal of the 12th transmembrane helix (TMH12) (Krishnamurthy et al. 1998). Δ*CDR1* was expressed in both *S. cerevisiae* and the baculovirus/insect cell system. In *S. cerevisiae*, ΔCdr1 exhibited no changes in ATPase activity in response to drug stimulation and lost efflux activity for cycloheximide and anisomycin, indicating that TMH12 is essential for maintaining the structural integrity required for drug recognition and/or transport (Krishnamurthy et al. 1998). In contrast, ΔCdr1 expressed in the baculovirus/insect cell system showed a 1.8-fold increase in ATPase activity upon stimulation with rhodamine 123, demonstrating differences in ATPase activity behaviour across expression systems. These differences may reflect variations in the lipid composition and properties of the host cell membranes, or differences in posttranslational modifications in the two expression systems.

Pichia pastoris

Although there are no reports of using *Pichia pastoris* for heterologous expression of Cdr1, this yeast expression system offers several advantages. These include straightforward genetic manipulation, simple and cost-effective nutrient requirements, rapid growth, high yield, and efficient secretion of soluble proteins. In *P. pastoris*, recombinant gene expression involves cloning the target gene into an expression vector under the control of the alcohol oxidase (AOX) promoter, followed by integration into the *P. pastoris* host genome. *Pichia pastoris* is a methylotrophic yeast capable of using methanol as its sole carbon source. In the presence of methanol, transcription of AOX is induced, allowing for growth on methanol. In *S. cerevisiae*, expressed proteins are often excessively N- and O-glycosylated, which can influence the immunogenicity of the proteins. In contrast, *P. pastoris* often performs appropriate glycosylation and enables the production of recombinant proteins with high yields (Karbalaei et al. 2020). Perhaps it is the high level of expression of correctly localized Cdr1 achieved in *S. cerevisiae* that explains why *P. pastoris* has not been used to study Cdr1.

Homologous expression in *C. albicans*

Homologous expression in *C. albicans* offers the advantage of maintaining the native lipid environment and membrane composition of Cdr1, thereby minimizing potential experimental artifacts. The widely used reference strain SC5314, along with its *URA3*-deleted derivatives, including CAF2-1 (Δ*ura3*::*imm434*/*URA3*), CAF4-2 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*), and CAI-4 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*) (Fonzi and Irwin 1993) have commonly been employed for homologous expression studies, in which Cdr1 expression and localization has been visualized through GFP-tagging. An early study utilizing GFP-tagged Cdr1 for expression and localization purposes was conducted in strain RM1000, a derivative of CAI-4, where *CDR1-GFP* was inserted at the native *CDR1* locus via homologous recombination (Hernaez et al. 1998). In addition, to explore how membrane composition influences Cdr1 function, *CDR1-GFP* was introduced into a *C. albicans erg11* mutant, allowing insights into the relationship between altered sterol composition and Cdr1 activity (Suchodolski et al. 2019). The lack of ergosterol, and abnormal sterol deposition, in the *erg11* mutant resulted in reduced plasma membrane permeability. Cdr1 was expressed at the same level in the *erg11* mutant as in the wild type strain, but R6G efflux was lower and Cdr1was mislocalized to the vacuole (Suchodolski et al. 2019). The expression level of *CDR1* in *C. albicans* is influenced by various environmental factors, including temperature, energy sources, and exposure to a range of chemical compounds. These elements can modulate the transcriptional activity of the gene, reflecting the adaptive mechanisms of *C. albicans* to environmental changes and stress conditions (Krishnamurthy et al. 1998). Doxorubicin (adriamycin) was found to upregulate *CDR1* expression in *C. albicans*, as evidenced by increased *CDR1* mRNA levels and enhanced fluorescence from a Cdr1–GFP fusion protein (Kofla et al. 2011). Human steroid hormones including oestradiol and progesterone, fluphenazine (de Micheli et al. 2002) and the recently developed fluphenazine derivative CWHM-974 (Miron-Ocampo et al. 2023) are also strong inducers of *CDR1* expression. A detailed list of known and potential inducers of Cdr1 expression, and whether these compounds are potential or known efflux pump substrates or inhibitors of Cdr1, is provided in Table S6.

Candida albicans is diploid meaning it contains two copies of most genes, and for Cdr1 the alleles have been referred to as *CDR1A* and *CDR1B* (Holmes et al. 2006). Genetic manipulation of *C. albicans* often involves genomic integration of auxotrophic markers such as *URA3* via homologous recombination for the selection of transformants. The use of 5-fluoroorotic acid (5-FOA) for *URA3* marker recycling, dominant selection markers like *SAT1* or *NAT1* recycled with the site-specific recombinase FLP, or the more recently developed CRISPR-Cas9 systems are widely employed for the efficient generation of homozygous *C. albicans* deletion strains (Reuss et al. 2004, Shen et al. 2005, Min et al. 2016). The homozygous disruption of Cdr1 was first achieved in the CAF4-2 strain background, resulting in DSY449 (Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG*) (Sanglard et al. 1996). This strain was subsequently used to reintroduce one *CDR1* allele at the *CDR1* locus that, as expected, restored fluphenazine resistance (Zhu et al. 2011). Later studies developed a CAI-4/ΔCDR1 strain with a FLAG-tagged Cdr1 under the control of three different promoters (*ACT1*, *MAL2*, and *MET3*) integrated at the *RP10* locus—a reliable site for stable gene expression in *C. albicans*. Another construct involved directly FLAG-tagging one allele of *CDR1* in the CAI-4 background (Umeyama et al. 2002). Strains containing the *ACT1* or *ADH1* promoter to drive the constitutive expression of *CDR1* have proved useful to study the synergistic effects of new antifungals with existing drugs, since they avoided the influence of drug-induced up- or downregulation of *CDR1* expression (Urai et al. 2014, Wang et al. 2022). To reduce background multidrug resistance, strains such as STY31 lacking *CDR1* and *CDR2*, and DSY1050 lacking *CDR1*, *CDR2*, and *MDR1* were used as host strains to express *CDR1* through genomic integration or on a multicopy plasmid, respectively (Basso et al. 2010, Tsao et al. 2016). It is worth noting that *C. albicans* STY31 was derived from an azole-resistant clinical isolate, 5674, that overexpressed *CDR1* due to a homozygous N972D mutation in *TAC1*, a zinc cluster transcription factor acting on *CDR* genes (Znaidi et al. 2007). A homologous overexpression system taking advantage of this gain-of-function mutation in Tac1 was constructed by the deletion of *MDR1* in the STY31 strain, resulting in the lack of three major multidrug transporters Cdr1, Cdr2, and Mdr1 (Banerjee et al. 2016). The system was validated by the increased resistance to pump substrates when *CDR1* was reintroduced at the *CDR1A* locus, and demonstrating the efficient efflux of R6G and Nile Red, the effectiveness of pump inhibitors and the proper localization of Cdr1 to the cell surface. The Cdr1 variants Cdr1-L529A and Cdr1-

V532A (located in TMH1) and Cdr1-C1294A (located in TMH9) which, when expressed in *S. cerevisiae*, were previously shown to be critical for substrate binding (Rawal et al. 2013), were also constructed in *C. albicans* to validate the homologous expression system (Banerjee et al. 2016). The Cdr1-L529A and Cdr1-V532A variants conferred phenotypes on the *C. albicans* host consistent with those conferred on *S. cerevisiae*. The Cdr1-C1294A variant, however, demonstrated contrasting phenotypes in the two expression systems, suggesting potential differences in the functional impact of the mutation depending on the different host membrane environments. This homologous expression system may provide a powerful platform for functional and structural analysis of drug resistance MPs in *C. albicans* that circumvents artifacts from heterologous expression systems.

Candida albicans, however, is not ideal for functional analysis of MPs. The diploid nature of *C. albicans* makes gene integration or knockout more challenging. Also, the lack of strong, constitutive promoters limits the ability of *C. albicans* to achieve consistently high expression levels with minimal influence by environmental conditions. Furthermore, when under stress from antifungal drugs, *C. albicans* can also undergo chromosomal rearrangements, such as chromosome amplification or trisomy, to regain drug resistance and tolerance (Yang et al. 2023). These genomic changes can complicate functional analysis due to gene dosage effects and altered drug responses. Homologous expression may still be useful, however, for structural studies by maintaining Cdr1 in its native membrane environment.

Importance of the lipid environment for the correct function/structure of Cdr1

More than a quarter of the average genome encodes MPs, and MPs account for more than half of all current drug targets (Yildirim et al. 2007, Bakheet and Doig 2009). To develop treatments for the many clinical problems caused by microbial MPs and dysfunctional human MPs, it is essential to have access to high-resolution structures that most accurately reflect the 'true' structure of those proteins in their native membrane environment. Structure-directed drug design can overcome Cdr1-mediated drug resistance, but the MP structure needs to be correct. We believe that many ABC MP structures (Locher 2016) including the structures of *S. cerevisiae* Pdr5 (Harris et al. 2021) and *C. albicans* Cdr1 (Peng et al. 2024), are inaccurate (Guo 2020), as they were obtained with detergent-solubilized MPs stripped of their native lipid-microdomains (Spira et al. 2012, Douglas and Konopka 2016, Malinsky and Opekarova 2016)—which are unique and essential for functional MP–lipid complexes which have recently been termed memteins (Overduin and Esmaili 2019) (i.e. membrane proteins).

The exponential increase in the number of solved MP structures (Li et al. 2021) over the last two decades has been due to the development of advanced MP analysis technologies, increased access to high quality detergents, lipids, and optimized MP crystallization buffer suites, remote access to advanced X-ray and cryo-EM facilities, and the rapid development of high-resolution imaging technologies and open-access bioinformatics platforms. Most MP structures to date are for MPs extracted with detergent, which removes native membrane lipid components crucial for their structural and functional integrity (Sharom 2014, Qiu et al. 2018, Overduin and Esmaili 2019, Guo 2020). Although molecular dynamics simulations of MPs in lipid bilayers (Stansfeld et al. 2015, Marrink et al. 2019) has tremendous potential to improve our understanding of how MPs interact with the phospholipid bilayer (Corradi et al. 2019), more direct experimental evidence is needed to confirm MP structure and function in the native membrane bilayer (Marrink et al. 2019). Recent experimental evidence led to the proposal of a unique substrate entry gate for Cdr1 between TMH1 and TMH11 with G521, at the centre of TMH1 acting as the gatekeeper residue that determines whether Cdr1 efflux pump substrates or inhibitors can enter the transporter (Niimi et al. 2022). The cryo-EM structures for Pdr5 (Harris et al. 2021) and *C. albicans* Cdr1 (Peng et al. 2024) were consistent with this entry gate hypothesis. However, there are a number of concerns about the accuracy of these structures and many fundamental questions remain unanswered: Are Cdr1 efflux pump substrates directly pumped into the supernatant ('vacuum cleaner' model) or are they flipped from the inner to the outer lipid bilayer leaflet and then diffuse into the extracellular space ('flippase' model) as proposed for Pdr5 (Raschka et al. 2022)? How do the lipid composition and the physical properties of the plasma membrane microdomain surrounding Cdr1 influence its structure and function?

The discovery that small organic amphipathic polymers such as styrene maleic acid (SMA) polymers can extract native MP–lipid bilayer complexes (SMA–lipid particles; SMALPs) initiated a new era in MP analysis (Overduin and Esmaili 2019, Brown et al. 2021). Since their discovery in 2009 (Knowles et al. 2009), significant progress has been made with a number of commercially available SMA polymer derivatives. Some have led to high resolution crystal and/or cryo-EM MP–SMALP structures revealing features not visible in structures of the same MP purified with detergent (Yoder and Gouaux 2020, Brown et al. 2021). SMALP purification applied to Cdr1 may, therefore, reveal the structure of the first 86 N-terminal residues and the region between TMD1 and NBD2 not visible in the Pdr5 structures (Harris et al. 2021) but clearly quite important for Cdr1 function (Tsao et al. 2016).

Proposed model for Cdr1 efflux pump function

Tremendous progress has been made in understanding the structure and transport function of Cdr1 since its discovery 30 years ago. Figure 4 provides cartoon models of the inside-open and outside-open conformations of Cdr1 based on the first fungal PDR transporter structure solved, *S. cerevisiae* Pdr5 (Harris et al. 2021). ABCG transporters like Cdr1 have two N-terminal NBDs and two C-terminal transmembrane domains (TMDs) (Fig. 4A) with an atypical inverted ABC transporter topology $(NBD\text{-}TMD)_2$ (Dean et al. 2001). Fungal PDR transporters also have asymmetric CNBSs comprising the Walker A and B motifs from one NBD and the ABC signature motif from the other NBD. The noncanonical CNBS1 (Walker A1-B1-ABC2) binds ATP (magenta sticks; Fig. 4A) at all times during the transport cycle. The recently discovered fungal PDR transporter-specific linker domains, LD1 and LD2 (light green; Fig. 4A), (Harris et al. 2021) and CNBS1 provide tight interactions between amino acids and ATP that ensure that the rear of the transporter between TMH5 and TMH7 is closed at all times. Binding of ATP (green sticks; Fig. 4A) at the catalytically active canonical CNBS2 (Walker A2-B2-ABC1) just underneath the substrate entry gate causes large conformational changes in the TMDs that force substrates through a hydrophobic exit valve (Fig. 4B) into the extracellular space or possibly the outer lipid bilayer (red arrows; Fig. 4A). After substrate release, ATP hydrolysis at CNBS2 returns the transporter to the inside open conformation. The first

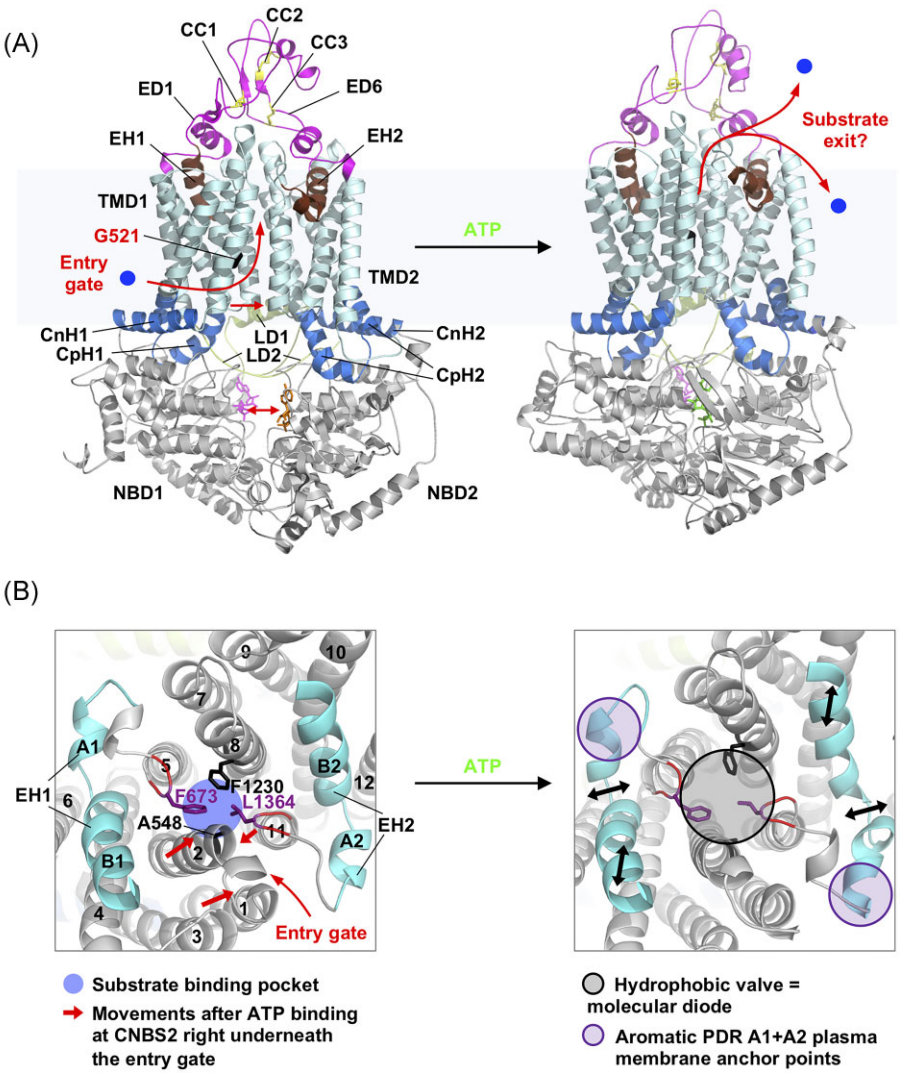


Figure 4. Models for the *C. albicans* Cdr1 efflux pump structure and function. (A) Cartoon models of the inside-open (left) and outside-open (right) conformations of Cdr1 based on *S. cerevisiae* Pdr5 (Harris et al. 2021). Cdr1 has two NBDs (grey), two TMDs (cyan), and two extracellular domains (EDs; magenta) with three disulphide bonds (CC1, CC2, and CC3) that stabilize the structural and functional integrity of Cdr1. ATP-binding (green sticks) at the catalytically active CNBS2, underneath the substrate (blue circles) entry gate between TMH1 and TMH11 with the G521 gatekeeper residue (black) at the centre of TMH1 causes large conformational changes of Cdr1 that are transmitted from the NBDs to the TMDs via two conserved connecting (CnH1 and CnH2) and two coupling (CpH1 and CpH2) helices (colored blue). The exchange of ADP (orange) with ATP at canonical CNBS2 brings the two NBDs close together. This rigid movement of the two NBDs causes the entry gate above to close and forces substrates into the extracellular space or the outer lipid bilayer (red arrows) through a hydrophobic valve just above the substrate-binding pocket. ATP hydrolysis at CNBS2 returns the transporter back to the inside open conformation. (B) Cartoon models of the inside-open (left) and inside-closed (right) conformations of the TMDs viewed from the top with the two EDs removed for clarity of view. Individual TMHs are numbered from 1 to 12 (left). The PDR transporter-defining PDR motifs (PDR A1, A2, B1, and B2; Lamping et al. 2010) form two elbow helices (EH1 and EH2; cyan) embracing the four centrally located TMHs (2, 5, 8, and 11) near the top of the transporter. ATP binding at CNBS2 causes closure of the entry gate with the red arrows indicating notable movements between TMH1, −2 and −11 at the front of the transporter. This movement forces substrates to exit through the hydrophobic valve (grey circle on the right) just above the substrate binding pocket (blue circle on the left) between TMH2, −5, −8, and −11. The hydrophobic F, L, and A amino acids that are part of this valve are shown as magenta and black sticks. Preliminary evidence suggests that conserved aromatic residues near the N-terminus of the PDR A1 and A2 motifs (purple circles) provide critical plasma membrane anchor points for the unidirectional expulsion of substrates through the hydrophobic exit valve.

ABCG transporter structures solved were for the human cholesterol transporter ABCG5/G8 (Lee et al. 2016) and the human multidrug efflux transporter ABCG2 (Taylor et al. 2017). These structures revealed for the first time the positioning of the PDR transporter specific motifs (PDR A, PDR B, EL6 motif, and EL6 helix) discovered in 2010 (Lamping et al. 2010). Others have called these unique helices TMH5B and 5C (Taylor et al. 2017) or reentry helix (Khunweeraphong et al. 2019). We prefer the term elbow helices (i.e. EH1 and EH2; brown and cyan in Fig. 4A and B, respectively) because, like elbows, EH1 and EH2 dip halfway down into the plasma membrane lipid bilayer with the upper (PDR A1 and A2) and lower (PDR B1 and B2) arms each connected by conserved proline linkers (Lamping et al. 2010). Another common feature of ABCG transporters is the hydrophobic valve at the top of a centrally located substrate binding pocket (Fig. 4B), first described for ABCG2 (Manolaridis et al. 2018, Khunweeraphong et al. 2019) and more recently for *S. cerevisiae* Pdr5 (Alhumaidi et al. 2022). This has led to the efflux pump model presented in Fig. 4: ATP bind-

ing at CNBS2 causes closure of the entry gate [straight red arrows highlight important TMH- and NBD-movements (Fig. 4A and B) upon ATP-binding] and forces substrates to exit through the hydrophobic valve (grey circle; Fig. 4B) just above the substrate binding pocket (blue circle; Fig. 4B) between TMH2, −5, −8, and −11. The F, L and A amino acids that are part of this valve are shown as magenta and black sticks (Fig. 4B). The two conserved EHs provide the hydrophobic valve with the necessary flexibility to allow differently sized substrates to 'squeeze' through the transporter while preventing any possible leakage through this valve. We have evidence to suggest that the conserved aromatic residues near the N-terminus of PDR A1 and A2 (purple circles; Fig. 4B) (Lamping et al. 2010) behave like 'hands' that 'anchor' Cdr1 to the outer plasma membrane leaflet, which is critically important for the unidirectional expulsion of substrates through this hydrophobic exit valve.

Conclusions and future research

Well-characterized heterologous expression systems are powerful tools for achieving high levels of expression that can confer strong phenotypes in the presence of reduced background activities. They do have drawbacks, however. These include potentially different codon assignment, different posttranslational modification, and different membrane environments. There is increasing evidence that MP function is affected by the composition and dimensions of the membrane in which they are located, and that within a particular membrane there are many different domains with different lipid compositions (Douglas and Konopka 2016). The situation for Cdr1 is complicated further by its function as a lipid flippase (Dogra et al. 1999) meaning that overexpression of Cdr1 can affect membrane composition/asymmetry and hence function. Future research should focus on homologous expression and structural analysis of the protein in its native lipid environment. Fusing affinity tags to Cdr1 could enable purification of Cdr1 away from other proteins with similar function, and low levels of expression could minimize changes to the membrane composition and architecture.

Data availability

All plasmids presented in Figs 2 and 3 are available from Dr Erwin Lamping (erwin.lamping@otago.ac.nz) upon request.

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Supplementary data

Supplementary data is available at *FEMSYR Journal* online.

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