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REVIEW

Unravelling bacterial virulence factors in yeast: From identification to the elucidation of their mechanisms of action

ZhenPei Wong[1] · Eugene Boon Beng Ong[1]

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Abstract

Pathogenic bacteria employ virulence factors (VF) to establish infection and cause disease in their host. Yeasts, *Saccharomyces cerevisiae* and *Saccharomyces pombe*, are useful model organisms to study the functions of bacterial VFs and their interaction with targeted cellular processes because yeast processes and organelle structures are highly conserved and similar to higher eukaryotes. In this review, we describe the principles and applications of the yeast model for the identification and functional characterisation of bacterial VFs to investigate bacterial pathogenesis. The growth inhibition phenotype caused by the heterologous expression of bacterial VFs in yeast is commonly used to identify candidate VFs. Then, subcellular localisation patterns of bacterial VFs can provide further clues about their target molecules and functions during infection. Yeast knockout and overexpression libraries are also used to investigate VF interactions with conserved eukaryotic cell structures (e.g., cytoskeleton and plasma membrane), and cellular processes (e.g., vesicle trafficking, signalling pathways, and programmed cell death). In addition, the yeast growth inhibition phenotype is also useful for screening new drug leads that target and inhibit bacterial VFs. This review provides an updated overview of new tools, principles and applications to study bacterial VFs in yeast.

Keywords Growth inhibition · Heterologous expression · Pathogenic bacteria · Virulence factors · Yeast · Saccharomyces cerevisiae

Introduction

Bacterial virulence factors (VFs) are molecules that enable the bacteria to establish infection and cause disease in their hosts. VFs function as adhesins, invasins, colonisation factors, antiphagocytic factors, toxins, immune response inhibitors, autotransporters, and proteases either individually or together at different stages of bacterial infection in the host organism (Leitão 2020). Identifying bacterial VFs is the first step to understanding bacterial pathogenesis to enable the development of preventive and therapeutic strategies to combat bacteria-mediated disease (Davey and Valdivia 2020; Ahmad-Mansour et al. 2021). In the postgenomics era, the availability of bacterial and host genomic sequences, together with the application of bioinformatics, genomics, transcriptomics, and proteomics approaches have accelerated the discovery of bacterial VFs (Gonçalves Pessoa et al. 2019; Wang et al. 2019; Impens and Dussurget 2020; Rentzsch et al. 2020; Ali et al. 2022). The development and availability of various molecular toolboxes for genetically tractable models of host-pathogen interactions such as yeast (Valdivia 2004), amoebae (Amaro and Martín-González 2021), roundworm (Kaito et al. 2020), fly (Younes et al. 2020), moth larvae (Ménard et al. 2021), weed (Bozzaro 2019), zebrafish (Gomes and Mostowy 2020; Nag et al. 2020), mice (Tantengco and Yanagihara 2019), and hamsters (Miao et al. 2019) have expanded our means to study VFs and investigate bacterial pathogenesis using different host organisms.

Among the various model organisms, the budding yeast *Saccharomyces cerevisiae*, is one of the most popular and well-studied simple model for understanding the fundamental aspects of eukaryotic biology (Nielsen 2019). Yeast is easy to grow in the laboratory, can be maintained at a fraction

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✉ Eugene Boon Beng Ong
eugene@usm.my

[1] Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Penang 11800 USM, Malaysia

of the cost of other eukaryotic organisms, and is genetically tractable and amenable to high-throughput systems. As eukaryotes, yeast has provided a vast array of information on fundamental cellular processes that are highly conserved among all eukaryotes, including organelle biogenesis, cytoskeletal organisation (Akram et al. 2020), cell cycle control (Legrand et al. 2019; Vanderwaeren et al. 2022), membrane trafficking (Ma and Burd 2020), DNA metabolism (Gupta and Schmidt 2020), cell quiescence (Lee and Ong 2020; Sun and Gresham 2021), cell death (Carmona-Gutierrez et al. 2018; Galimov et al. 2019) and cell signalling (Chen and Thorner 2007; Morozumi and Shiozaki 2021). Humans and *S. cerevisiae* share about 2100 groups of orthologous genes, and 280 yeast genes are replaceable by human genes (Laurent et al. 2020). Approximately ~ 80% of yeast open reading frames (ORFs) have been annotated and verified, facilitating research involving the yeast model (Wong et al. 2023). In addition, many resources that have been developed for yeast such as protein chips (Zhu et al. 2000), deletion mutant libraries (Giaever et al. 2002), gene overexpressing strains (Gelperin et al. 2005; Sopko et al. 2006), the yeast GFP clone collection (Huh et al. 2003), and fully synthetic yeast genomes (Kutyna et al. 2022) are commercially available and can be directly adopted for experimental design and hypotheses validation. These allows the use of yeast in diverse medical fields such cancer biology (Ferreira et al. 2019), neurodegenerative disorders (Rencus-Lazar et al. 2019), ageing (He et al. 2018; Lee and Ong 2020), drug screening (Ong et al. 2011; Tavella et al. 2021), and microbial infectious diseases (Angrand et al. 2019; Sahaya Glingston et al. 2021).

It is unsurprising then, that the yeast heterologous expression system are widely applied to identify and characterise bacterial virulence proteins, especially type III, type IV and type VI secretion effectors, which are translocated directly across an additional host cell membrane into the host cell cytoplasm, enabling effective manipulation of host cellular processes. Several reviews have highlighted the contribution of the yeast model to bacterial virulence protein studies in the past (Valdivia 2004; Siggers and Lesser 2008; Curak et al. 2009; Popa et al. 2016a). In this review, we provide a more comprehensive and updated overview of the insights obtained from VF studies conducted in yeast to understand bacterial virulence. This review includes important recent studies on the tools, principles, and applications of the yeast model to study bacterial virulence proteins. In addition to that, our review discusses studies beyond the bacterial type III secretion effectors focused in the review by Popa et al. (2016a). Furthermore, there are also new relevant aspects that are discussed in detail, including the effector-effector suppression screen (to identify interactions between bacterial effectors), characterisation of host microtubule-modulating bacterial VFs in fission yeast, Ras- rescue screen (to identify membrane-associated bacterial VFs), TORC1 signalling pathway targeting-VFs, and the characterisation of cytolethal distending toxin in yeast which were not covered in past reviews. Thus, this review continues to highlight the strength of yeast as a useful model system to elucidate VFs involved in bacterial pathogenesis at the cellular level.

Identification of putative virulence factor with yeast growth inhibition assay

The inhibition effect of yeast growth caused by the cytotoxic effect of heterologously expressed bacterial VFs has been widely used as an initial study for the identification of bacterial VFs (Bosis et al. 2011; Zuo et al. 2024). Heterologous expression combined with growth inhibition phenotype allow studies of pathogens that are impossible, difficult, or risky to grow in the laboratory because it only requires the cloning of the pathogen's open reading frame (ORF) into expression vectors (Fig. 1a). Several factors can affect the yeast growth phenotypes caused by heterologous expression of the virulence protein, including the inducible promoter used in the expression system, the copy number of the virulence gene, the epitope tag used to verify protein expression, and the yeast strain itself (Siggers and Lesser 2008; Salomon et al. 2012). The addition of stressors such as caffeine to the media to uncover bacterial VFs that do not inhibit yeast growth in standard laboratory conditions (Siggers and Lesser 2008; Popa et al. 2016a) is discussed further in the 'MAPK signalling pathway' section.

There are two main methods (qualitative or quantitative) to monitor yeast growth after the expression of bacterial VFs. The first and most widely used method is the serial dilution spotting assay. The spotting assay is a convenient assay widely used to detect qualitative differences in VF-expressing yeast growth in the presence and absence of the inducers or stressors. Saturated yeast cultures are serially diluted and seeded on non-inducing and inducing, or chemically treated agar, and the difference between the size of the spots are compared after incubation (Bankapalli et al. 2015; Coronas-Serna et al. 2020). While the serial dilution spotting assay may be a quick, visual format for preliminary screening, this method provides an initial assessment of the severity of the toxic effect of the virulence protein (Rangel et al. 2019). Another method is the measurement of the optical density of liquid culture in a conventional and 96-well format liquid growth assay which can be used to quantify the exact growth inhibition effect of the VFs on yeast (Slagowski et al. 2008; Sukumaran et al. 2011). Alternatively, quantitative measurement of growth inhibition can also be achieved by determining the viability of yeast cells using the traditional

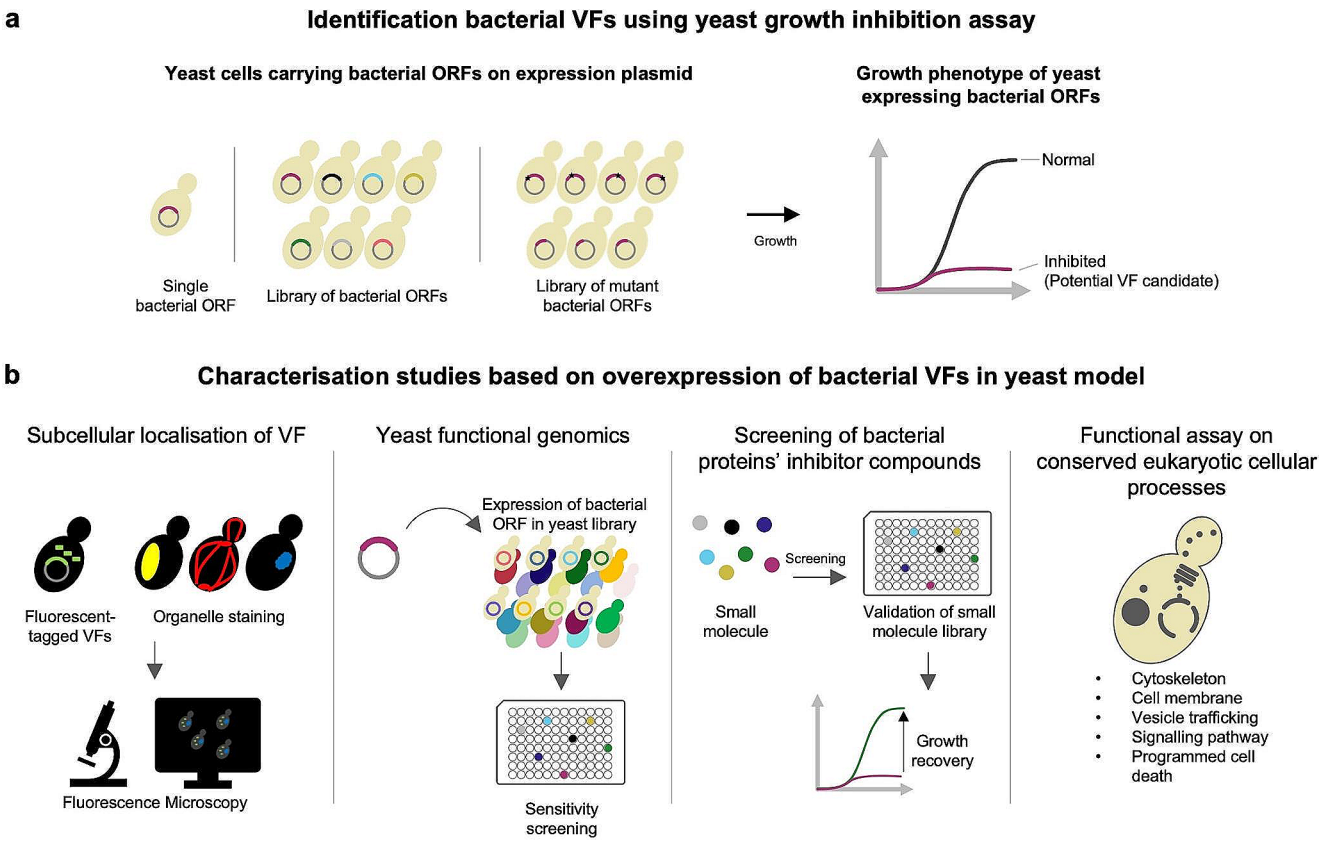

Fig. 1 Studies of bacterial virulence factors (VF) through the growth inhibition effects caused by heterologous expression of bacterial proteins. (**a**) The growth inhibition assay has been used to screen potential candidate VFs by investigating the yeast growth change caused by heterologous expression of bacterial proteins in yeast cells from a single assay to a high-throughput format. (**b**) The yeast strains that inhibited yeast growth will be selected for further characterisation studies such as subcellular localisation studies under microscopy, yeast functional genomics studies, functional assay on conserved eukaryotic cellular processes and screening of bacterial proteins' inhibitor compound

colony-forming unit (cfu) counting method (Salomon et al. 2012), or live-death cell staining which requires microscopy (Kwolek-Mirek and Zadrag-Tecza 2014).

The rationale of identifying VF candidates via the yeast growth inhibition phenotype is based on the premise that heterologously expressed bacterial VFs often target key cellular processes conserved among eukaryotes, and they elicit similar physiological responses in yeast and plant/animal host cells, resulting in suppression of yeast growth (Valdivia 2004; Siggers and Lesser 2008) (Fig. 1b). For example, *Shigella* effector proteins VirA, IpgD, IpgB1, IpgB2 and OspF which are known to target microtubules, inositol phosphate signalling, G-protein signalling and mitogen-activated protein kinase (MAPK) in human cells respectively, were also found to target the same proteins in yeast, which eventually inhibited yeast growth (Slagowski et al. 2008). Therefore, many bacterial VFs including, both known and unknown VFs, have been identified using the yeast growth inhibition assay (summarised in Table 1). Additionally, expression of mutated gene variants of bacterial VFs in yeast can also identify functional domains, motifs, and residues which are responsible for the bacterial VFs' activities that resulted in yeast growth inhibition (Coronas-Serna et al. 2020; Peng et al. 2020; Ratu et al. 2021; McCaslin et al. 2023).

Although the yeast growth inhibition assay is a rapid method to identify bacterial VFs, there are limitations to this assay. The assay may miss out on VFs if there is non-conservation between the targets of the mammalian/plant systems and yeast, or if bacteria-specific modifications for certain proteins are required which are absent in yeast, or if specific host factor/physiological conditions are absent in yeast to trigger the activities of bacterial VFs (Valdivia 2004; Slagowski et al. 2008; Bankapalli et al. 2017). Furthermore, overexpression of bacterial housekeeping proteins that are not related to virulence can also interfere with conserved cellular processes and cause growth inhibition, resulting in the misidentification of VFs (Siggers and Lesser 2008). Therefore, all these limitations must be considered when using yeast as a model for bacterial VF studies.

Table 1 Identification of bacterial VFs through heterologous expression in yeast growth inhibition assays

Bacterial pathogens	Number of VF genes identified	Promoter	Copy number of gene	Yeast strains	Tag	Stressor	Monitoring	References
Pseudomonas syringae	16 out of 75 (Only NaCl: 3: Only sorbitol: 3)	GAL1	low copy	Y7092	C-terminal FLAG epitope	NaCl, Sorbitol	Spot dilution	(Lee et al. 2019)
	7 out of 27 (other 5 only in the presence of stressor)	GAL1-10	low copy	BY4741	c-myc tag	Sorbitol, NaCl, tunicamycin, heat stress	Spot dilution	(Salomon et al. 2012)
Aeromonas spp.	15 out of 21	GAL1	low copy	BY4741	N-terminal 7× hemagglutinin tag	NaCl, caffeine	Spot dilution	(Rangel et al. 2019)
Ralstonia solanacearum	5 out of 33 (Only NaCl: 3)	GAL1	high copy	BY4741	-	NaCl	Spot dilution	(Zheng et al. 2019)
Wolbachia spp.	8 out of 47 (Only in $ZnCl_2$, caffeine: 5)	GAL1 (hormone β-oestradiol as inducer)	high copy	BY4742	N-terminal Xpress™ epitope	$ZnCl_2$, caffeine	Spot dilution, Western blot to check expression	(Carpinone et al. 2018)
Wolbachia pipientis	5 out of 84 (Only in nocodazole and/or caffeine: 7; Only in sorbitol and/or NaCl: 2)	GAL1	low copy	BY4741	N-terminal GFP	nocodazole, sorbitol, NaCl, caffeine	Liquid growth (OD)(z-score)	(Rice et al. 2017)
Salmonella Typhi; Salmonella Typhimurium	93 out of 4600; 27 out of 1600	GAL1	high copy	1783	N-terminal GST	-	Spot dilution	(A. Alemán et al. 2009)
Legionella pneumophila	10 out of 1750	GAL1	low copy	EGY48	N-terminal B42 activation domain and an HA tag	-	Spot dilution	(Campodonico et al. 2005)
Anaplasma phagocytophilum	1 out of 33	GAL10	high copy	S288C	C-terminal GFP tag	-	Liquid and solid growth assays	(Sukumaran et al. 2011)
Leptospira interrogans	9 out of 288	CUP1	high copy	MLC30M	C-terminal 6x HIS tag, FLAG tag	-	Liquid growth assay, spot dilution	(Lai et al. 2022)
Candidatus Liberibacter	1 out of 15	GAL1	low copy	W303-1 A	C-terminal V5 epitope, 6x HIS tag	-	Spot dilution assay	(Zuo et al. 2024)

Subcellular localisation of VFs in yeast by fluorescence microscopy

Although yeast growth inhibition assays can identify bacterial VF candidates based on their growth-inhibiting phenotype, they cannot reveal the functions or cellular targets of the VFs. Thus, subcellular localisation patterns of bacterial VFs expressed in yeast can reflect their localisation in the host cell during infection and also indicate their molecular targets. To visualise the heterologously expressed bacterial proteins in yeast cells, visible markers such as fluorescent tags are fused with the bacterial ORFs at the C- or N-terminals and observed using fluorescence microscopy. The green fluorescent protein (GFP) is the most widely used tag, either singly or together with other reporter proteins such as Discosoma Red (DsRed), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and mCherry (Sakalis et al. 2014; Rodriguez-Escudero et al. 2016; Bankapalli et al. 2017). Additionally, organelle-specific stains are also essential for the identification of the expressed protein in a specific subcellular compartment. For example, 4', 6-diamidino-2-phenylindole (DAPI), a DNA binding dye is used for nuclear-staining; Rhodamine-phalloidin stains the actin cytoskeleton; FM4-64 stains the vacuole; MitoTracker and methyl pyridinium iodine (DASPMI) stain mitochondria; DiOC6 (3) stains mitochondria, endoplasmic reticulum (ER) and Golgi apparatus; Trypan blue, Calcofluor white and concanavalin A stain the cell wall, and specific antibody markers are used for visualisation of particular compartments (Hasek 2006; Liu et al. 2022; Zhao and Guo 2023).

Moreover, certain staining dyes can also function to determine cell viability; for example, FUN-1, which stains cylindrical intravacuolar structures, is used to estimate metabolic activity. When stained with FUN-1, a live cell with metabolic activity contains cylindrical red-fluorescent structures in its vacuoles, while dead cells with little or no metabolic activity exhibit diffuse green fluorescence from the whole cytoplasm (Kwolek-Mirek and Zadrag-Tecza 2014).

Localisation patterns of bacterial virulence proteins in yeast can provide valuable insights into the molecular mechanism of virulent proteins. For example, the effector proteins Lpg0634, Lpg1751 and Ceg19 of *Legionella pneumophila* exhibited the same subcellular localisation pattern in yeast and in Hela cells with constitutively active Rab5 bound to the endocytic vesicles of both cell types (Weigele et al. 2017). The EPIYA (Glu-Pro-Ile-Tyr-Ala) motifs, which are responsible for manipulating host cell signalling by promiscuously interacting with multiple SH2 domain-containing proteins, were shown to be not essential for membrane localisation of *Lawsonia intracellularis* LI0666 in both *S. cerevisiae* and in mammalian cells (Chen et al. 2022). The *Salmonella enterica* serovar Typhi *Salmonella* invasion protein A (SipA), which could bind to yeast actin filaments, was shown to mediate actin-binding activity and the uptake of the *S.* Typhi bacterium into host cells (Lesser and Miller 2001). These examples show that subcellular localisation findings of bacterial VFs in yeast may be used to extrapolate their roles in their target hosts, but considerations must be taken to avoid the misidentification of subcellular localisations. Fusion tags can sometimes affect the expression and/or stability of the bacterial VFs, which then influence their phenotypic changes in yeast (Slagowski et al. 2008). Additionally, microscopy images may not always accurately reflect the actual interaction between bacterial VFs and their targeted organelles, thus careful detailed analyses are needed to validate the localisation results. However, the continuous development of more accurate methods such as time-resolved fluorescence microscopy can now be used for multidimensional imaging screening of the dynamic host-pathogen cross talk (Sanchez et al. 2022).

Yeast genome-wide screens to reveal VF functions and identify their targets

As the budding yeast *S. cerevisiae* is one of the major model organisms for understanding cellular and molecular processes in eukaryotes, the yeast functional genomic screen has been widely adopted to elucidate fundamental processes of cell biology, metabolism, and genetics. Yeasts have a relatively small genome which are well annotated (Wong et al. 2023). In this section, we describe how the cellular targets of bacterial VFs in yeast can be identified based on the expression of bacterial VFs in different yeast genomic libraries such as the yeast deletion library, yeast ORF-overexpressing library, and bacterial ORF expressing library (Fig. 2).

Synthetic genetic array (SGA) is a systematic method that introduces a query mutation to an array of approximately 5000 viable yeast gene-deletion mutants to construct double yeast mutants and allow large-scale mapping of synthetic genetic interactions (Tong and Boone 2006).

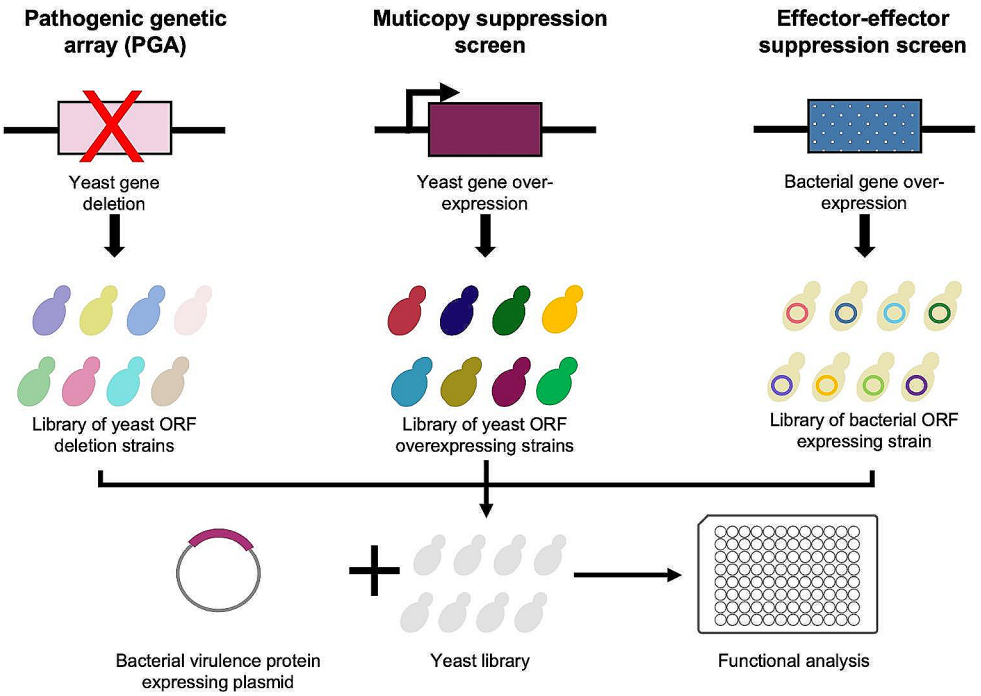

Fig. 2 Bacterial-host protein interactions can also be studied with the help of yeast genome libraries such as deletion library, overexpression library and bacterial ORF-expressing library. Upon transformation of the bacterial protein-expressing plasmid into individual yeast strains of the library, phenotypic effects such as growth can be evaluated in a high-throughput format

A combination of mutations in two genes results in either a negative genetic interaction referring to reduced fitness, synthetic lethality in extreme cases, or a positive genetic interaction referring to a reduced fitness defect in the same or two parallel pathways (Holstein et al. 2018). Large-scale genome-wide SGA screens have provided global genetic interaction profiles in the yeast genome and references to predict the function of uncharacterized genes since the genes within the same pathway tend to show very similar genetic interaction profiles (Costanzo et al. 2016). On the other hand, Pathogenic Genetic Array (PGA) is an SGA-like technology that enables high-throughput genetic screens to identify conserved cellular processes targeted by bacterial VFs. PGA screens the yeast genes that exacerbate or rescue the growth defect caused by bacterial VFs (Alto et al. 2006; Kramer et al. 2007; Lee et al. 2019). Based on this approach, several targeted cellular processes of bacterial VFs had been identified as listed in Table 2.

Previously, Kramer et al. systematically screened a collection of 4750 viable yeast deletion strains for mutants hypersensitive to the expression of the *Shigella* type III secretion effector OspF. There are 83 deletion strains hypersensitive to OspF that were identified to be involved in cell wall biogenesis with the aid of statistical data mining on synthetic lethal interaction data (Kramer et al. 2007). Bosis et al. optimised the yeast deletion hypersensitivity screen from Kramer et al. by developing an array of 90 yeast deletion strains fitted into a single 96-well plate that covers most (69%) of the yeast genetic interactions with less than 2% of the deletion strains in the entire yeast collection (Bosis et al. 2011). The array identified 13 genes that shared a synthetic lethality partner with OspF and were involved in processes related to cell wall biogenesis. There are 8 of 13 congruent genes also found by Kramer et al. (Kramer et al. 2007; Bosis et al. 2011). This 90 yeast deletion strains array simplified the process, reduced cost, and allowed the analysis of more virulence proteins in a short time with more repetitions of analysis. Using this approach, 12 genes congruent to *Xanthomonas campestris* type III secretion effector, XopE2 were identified to affect the yeast cell wall and the stress response of the endoplasmic reticulum (Bosis et al. 2011).

Conversely, the yeast multicopy suppressor screen, with a similar approach to SGA, was used to identify the conserved cellular process in eukaryote cells targeted by bacterial VFs based on the available collection of annotated yeast ORF-overexpressing strains instead of the yeast deletion strains collection (Gelperin et al. 2005; Sopko et al. 2006) as shown in Table 3. This approach is based on the hypothesis that overexpression of the targets of virulence proteins will suppress yeast growth defects caused by virulence proteins.

Apart from identifying the interaction between bacterial VFs and host targets, the yeast model system can also be used to identify the bacterial effector-effector interactions for a better understanding of the pathogen's molecular mechanisms during pathogenesis. The interaction between two effectors can be identified by the co-expression of two effectors in a yeast cell. For example, *L. pneumophila* LubX was demonstrated to be a meta-effector because the co-expression of LubX and SidH can suppress the growth-inhibiting effect of SidH in yeast (Quaile et al. 2015). Additionally, mutagenesis of LubX surface-exposed residues followed by functional screening in yeast identified the residue (Arg121) critical for LubX-SidH interactions, which were later confirmed by co-precipitation experiments. In another study, a systematic screen of all known *L. pneumophila* effector proteins for effector–effector interactions was performed by co-expression in yeast (Urbanus et al. 2016). More than 108,000 pairwise effector–effector genetic interactions between two libraries of ~330 type IV secretion effectors were co-expressed in *S. cerevisiae*. Effector-effector interactions were identified by selecting the suppressor for inhibition of yeast growth caused by overexpressed effectors. This approach rediscovered six known effector-effector antagonisms and identified an additional seventeen novel effector–effector suppression pairs, nine of which showed direct physical interaction with each other. Surprisingly, this approach also exposed the synergistic interaction between

Table 2 List of the bacterial VFs' studies involving pathogenic genetic array

Virulence protein	VF toxicity on yeast deletion mutant		Annotation	Reference
	Suppress	Hypersensitive		
IpgB2	3 (*Δbck1*, *Δslt2*, and *Δrm1*)	-	Activates the Rho1p GTPase Signalling	(Alto et al. 2006)
OspF	-	-	OspF acts to inhibit the CWI pathway	(Kramer et al. 2007)
OspB	81		Sensitize yeast to TORC1 inhibition	(Wood et al. 2022)
VepA	Δ*vma3*	-	Target V-ATPase subunit c	(Matsuda et al. 2012)
HopZ1a	137	53	Affect GTPase-mediated signal transduction	(Lee et al. 2019)
HopF2	132	73		
HopX1	-	88	Influence lipid metabolism	
CdtB	61	-	Induce DNA lesion, specifically DNA single-strand breaks	(Kitagawa et al. 2007)

Table 3 List of the bacterial VFs' studies involving multicopy suppression screen

Virulence protein	Suppressor identified by multicopy suppressor screen	Bacteria species	Reference
ExoS	Yeast Ras2p (a small GTPase).	*Pseudomonas aeruginosa*	(Arnoldo et al. 2008)
YopT	Cdc42 (Rho GTPase).	*Yersinia pestis*	(Shao et al. 2002)
SteC	Cdc42 (Rho GTPase).	*Salmonella Typhimurium*	(Fernandez-Piñar et al. 2012)
IpaJ	ADP-ribosylation factor (ARF)1p and ARF2p (small molecular mass GTPases); VPS15p (a phosphatidylinositol kinase required for yeast vacuole fusion).	*Shigella flexneri*	(Burnaevskiy et al. 2013)
BtpB	INM2, RBK1, and DOG2 (sugar or inositol phosphorylating/dephosphorylating enzymes); DOG2 (a 2-deoxyglucose-6 phosphate phosphatase); RBK1 (a putative ribokinase).	*Brucella abortus*	(Coronas-Serna et al. 2020)
CirA	Yeast Rho GTPase Rho1	*Coxiella burnetii*	(Weber et al. 2016)
Ceg14(Lpg0437)	Profilin (a protein involved in cytoskeletal structure in eukaryotes).	*Legionella pneumophila*	(Quaile et al. 2018)
AnkX	Small GTPase Ypt1 involved in membrane trafficking (Rab1 for mammalian cells); Bet1p, Sec22p, and Bos1p, three SNARES involved in ER to Golgi membrane transport.	*Legionella pneumophila*	(Tan et al. 2011)
LecE	Dgk1 (a diacylglycerol kinase enzyme)	*Legionella pneumophila*	(Viner et al. 2012)
OspB	Bre1p (E3 ubiquitin ligase)	*Shigella flexneri*	(Wood et al. 2022)

SidP and Lem14, which only inhibited yeast growth when co-expressed but not when they were individually expressed; interestingly a yeast-two hybrid assay showed that they do not interact physically (Urbanus et al. 2016).

Screening for small molecule inhibitors of bacterial VFs that suppress yeast growth inhibition

The yeast growth inhibition phenotype can also be used to identify anti-virulence molecules in drug discovery efforts by screening for compounds that suppress the growth inhibition effect caused by the expression of bacterial VF in yeast. Arnoldo et al. developed a yeast-based phenotypic assay that combines functional and chemical genomics screening to identify small-molecule inhibitors that can suppress toxicity caused by heterologous expression of the *P. aeruginosa* effector protein Exoenzyme S (ExoS). Six potential inhibitors were identified from a library of 56,000 small compounds based on the restoration of yeast growth from ExoS-mediated toxicity. One of them, exosin, specifically inhibited ExoS ADP-ribosyltransferase activity in vitro via competitive inhibition with NAD^+ substrate of ExoS. Exosin and its analogues exerted a protective effect on both yeast cells and mammalian cells against ExoS toxicity (Arnoldo et al. 2008). This approach was also used to screen for inhibitory compounds of another *P. aeruginosa* effector protein, ExoU (Kim et al. 2014). As a result, arylsulfonamides was identified as ExoU inhibitors although it was less potent to another known inhibitor, Pseudolipasin A, an inhibitor of ExoU phospholipase A_2 activity (Lee et al. 2007). However, a recent study showed that arylsulfonamides does not inhibit ExoU in vitro nor protected transfected mammalian cells from ExoU cytoxicity as in yeast cells, possibly due to the lack of host cofactor for arylsulfonamides activities in mammalian cells (Foulkes et al. 2021).

More recently, a similar strategy was used to identify drugs that restore the plant pathogen *Candidatus* Liberibacter asiaticus's (*C*Las) FlgI-mediated growth inhibition in yeast (Zuo et al. 2024). A total of 1663 compounds were tested against FlgI, and cyclosporin A was found to be able to restore the growth of FlgI-expressing yeast. However, the authors also found other false-positive hit compounds in their screen, which disrupted the heterologous expression system, suppressing protein production, rather than the direct inhibition of VF protein (Fig. 3). While this shows the limitations of such a screening system, it was not to be unexpected as the heterologous expression system relies on plasmid-based protein expression, therefore, further validations are required.

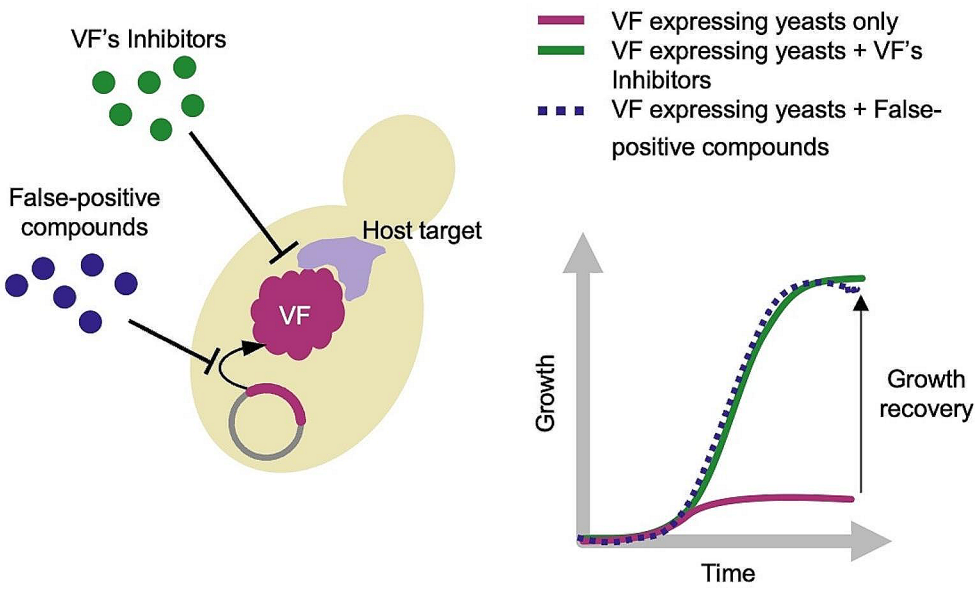

Fig. 3 Identification of small molecular inhibitors using yeast-based screening (Zuo et al. 2024). The effective compounds will be identified based on inhibition of bacterial VFs' activities on target molecule in yeast. However, there are possibilities of effective compounds as false-positive which inhibit the expression system instead of bacterial VFs' activities

Yeast functional assays for uncovering eukaryotic cellular structure and processes involved during pathogenesis

Alterations in yeast cytoskeleton by bacterial virulence proteins

During infection, the cytoskeletal rearrangement of the host cell promotes bacterial adhesion, invasion, structural support for bacteria-containing vacuoles, altered vesicular trafficking, actin-dependent bacterial movement, and pathogen dissemination (Caven and Carabeo 2019). It has been established that these dynamic cytoskeletal manipulations by bacterial VFs can be modelled in yeast.

Actin dynamics

The actin cytoskeleton is a dynamic network made up of actin polymers, which is highly conserved and essential for various fundamental cellular processes among eukaryotes (Akram et al. 2020). Manipulation and disruption of the host actin cytoskeleton are one of the most common strategies pathogenic bacteria use to drive cell infection by promoting bacterial cells uptake into the host cell or preventing their phagocytosis by macrophages (Stradal and Schelhaas 2018). The expression of bacterial VFs in yeast has been used to model the modulation of host actin cytoskeleton during bacterial invasion (Siggers and Lesser 2008; Popa et al. 2016a). For example, an ortholog of the chlamydial translocated actin recruiting phosphoprotein (TarP), CPn0572 is an essential VF for bacterial invasion by polymerising the host's actin. The expression of CPn0572 in both budding yeast and mammalian cells has been shown to be colocalised with actin patches, and distinctly thickened and extended actin cables (Zrieq et al. 2017). The DUF 1547 domain (amino acids 478 to 536), an actin-binding domain in CPn0572, is required for the association of CPn0572 with F-actin as shown in yeast cells and then in human cells to modulate actin polymerization and depolymerization which then impairs cell growth (Zrieq et al. 2017; Braun et al. 2019). Furthermore, an in vitro actin filament binding assay demonstrated that CPn0572 stabilises F-actin against actin-depolymerising agents by displacement of the F-actin destabilising protein, cofilin (Zrieq et al. 2017). This finding in *S. pombe* demonstrates that CPn0572 modulates yeast actin cytoskeleton. The modulation caused increased sensitivity to Latrunculin B, an actin-depolymerizing drug, and massive defects in cell morphogenesis and septum formation (Braun et al. 2019). Additionally, Braun et al. found the C-terminus of CPn0572 (aa 536 to 755) to have a second actin-modulating domain and a vinculin-binding site for host actin modulation in both yeast and human cell models (Braun et al. 2019).

The yeast model for actin modulation showed that bacterial VFs subvert host actin rearrangement to mobilize across cells and disrupt cellular signalling pathways (Siggers and Lesser 2008; Popa et al. 2016a). For example, expression of the Toll-interleukin-1 receptor (TIR) domain of *Brucella abortus* BtpB alone was sufficient to inhibit yeast growth by altering the polarity of the yeast actin cytoskeleton, blocking endocytosis, downregulating phosphorylation of all signalling kinases and disrupting energy metabolism in a yeast cell (Coronas-Serna et al. 2020). Similarly, the expression of *Escherichia coli* secreted proteins such as EspD, EspG, and Map in budding yeast caused growth inhibition by depolarising the actin cortical cytoskeleton, while

the expression of EspF altered yeast morphogenesis, signalling pathway and septin ring integrity (Rodríguez-Escudero et al. 2005). Likewise, the expression of a WAS(p)-family protein, *w*Bm0076 from *Wolbachia*, an endosymbiont of *Brugia malayi*, in budding yeast also caused growth inhibition by targeting the Arp2/3-activating protein, Abp1p, to disrupt eukaryotic actin dynamics and cortical actin patch formation (Carpinone et al. 2018; Mills et al. 2023).

In another example, the *P. aeruginosa* ExoY is an adenylate cyclase that breaks the microtubule and increases the permeability of the target cell for bacterial invasion after activation by F-actin (Cowell et al. 2005; Balczon et al. 2013; Belyy et al. 2016). The C-terminus of ExoY, especially the last nine C-terminal aa of ExoY, are crucial for toxicity in yeast, and its binding to F-actin in vitro contributed to its enzymatic activity (Belyy et al. 2018). The results from a yeast genetic screen and co-sedimentation assay showed that Asp25 of actin acts as a key residue for C-terminus ExoY–F-actin interaction, which was further confirmed by confocal microscopy (Belyy et al. 2016, 2018).

Microtubule

Some bacterial VFs also modulate the dynamics of the microtubule cytoskeleton by directly interacting with the tubulin αβ heterodimer or by recruiting cellular proteins that affect the dynamics of the microtubules (Radhakrishnan and Splitter 2012). The yeast model has demonstrated that the modulation of microtubules and growth cycle arrest by bacterial VFs promotes infection. For example, the *Chlamydia pneumoniae* CopN expression in both yeast and mammalian cells arrests the G2/M cell cycle due to disruption of spindle apparatus formation through disruption of the microtubule (Huang et al. 2008). In other examples, the *E. coli* EspG and *Shigella* homolog, VirA, can also disrupt the microtubule in yeast and mammalian cells, preventing coordination between the development of buds and nuclear division (Hardwidge et al. 2005; Rodríguez-Escudero et al. 2005; Slagowski et al. 2008).

Recently, a functional yeast-based screen was used to identify host microtubule-modulating bacterial VFs (Wevers et al. 2023). In the study, *S. pombe* was used to identify *C. pneumoniae* proteins that modulated the microtubule cytoskeleton. Thirteen chlamydial proteins were found to inhibit yeast growth, and increased the yeast's sensitivity to the microtubule destabilising drug thiabendazole and the microtubule inhibitor methyl benzimidazol-2-yl-carbamate. Subsequently, high-level expression of the 13 chlamydial genes in conditional-lethal tubulin mutant strains led to synthetic lethality. Furthermore, alterations in *S. pombe* interphase microtubules were also observed using a GFP-α-tubulin strain. One of the 13 chlamydial proteins, CPn0443, was found to be bound to microtubules in vitro, and co-localized partially with microtubules in vivo both in yeast and human cells (Wevers et al. 2023).

Alteration of host membrane structure and vesicle trafficking by bacterial virulence proteins

Certain bacterial VFs manipulate host membranes and the bacteria's trafficking to protect bacteria from host defence for bacterial survival, replication, and dissemination of pathogenic bacteria into host cells (Kostow and Welch 2023). Yeast has been used as a model for studying bacterial strategies to manipulate the host's membrane trafficking machinery, including alteration of the membrane, hijacking of various vesicle trafficking pathways, and escape from host defence mechanisms.

Bacterial VFs can manipulate the membrane by directly interacting with the membrane phospholipid. For example, the *Salmonella enterica* serovar Typhimurium SopF targets the host cell membrane through phospholipid interactions to promote the stability of the nascent *Salmonella*-containing vacuole (Lau et al. 2019). In yeast, the subcellular localisation of SopF is dependent on the activity of Mss4, a phosphatidylinositol 4-phosphate 5-kinase that generates phosphatidylinositol 4,5-bisphosphate, $PI(4,5)P_2$ synthesis for the organisation of the actin cytoskeleton and cell morphogenesis in *S. cerevisiae* (Lau et al. 2019). *Legionella pneumophila* LpdA, a palmitoylated phospholipase-D (PLD) triggers Golgi disruption in mammalian cells by modulating host cell phosphatidic acid (PA) level (Schroeder et al. 2015). LpdA also causes a lethal effect only in the yeast *dgk*1 deletion mutant and enhances the lethal effect of LecE by generating more PA as a substrate for PA phosphatase, which is activated by LecE (Viner et al. 2012). While VapA from *Rhodococcus equi*, which inhibits the maturation of *R. equi*-containing phagosomes and promotes intracellular bacterial survival, showed plasma membrane localisation by binding directly to PA when expressed in yeast (Wright et al. 2018). The expression of *L. pneumophila* effector protein, LegC7 in yeast caused vacuolar protein sorting defects (de Felipe et al. 2008), and specifically inhibited endosomal cargo delivery to the degradative vacuole (O'Brien et al. 2015). LegC7 interacted with the Emp46p/Emp47p ER-to-Golgi glycoprotein cargo adapter complex, disrupted ER morphology, and induced aberrant ER: endosome interactions, which were dependent upon endosomal VPS class C tethering complexes and the endosomal t-SNARE, Pep12p (Glueck et al. 2021).

Carboxypeptidase Y-invertase (CPY-Inv) overlay assay is a yeast assay that identifies bacterial VFs that can disrupt vesicle trafficking. The assay is performed by expressing a bacterial VF that is fused to a CPY-Inv hybrid protein

(Fig. 4a) (Shohdy et al. 2005; de Felipe et al. 2008). The CPY-Inv hybrid protein consists of a fusion between the first 50 aa of CPY which sorts signals for CPY trafficking from ER-Golgi to the vacuole and invertase which hydrolyses exogenous sucrose. Bacterial proteins that cause vacuolar protein sorting (VPS) defect can be detected by the formation of brown colonies in a yeast reporter strain NSY01 due to the hydrolysation of exogenous sucrose into glucose when cargo vesicles are blocked from reaching the vacuole, leading to the missorting of the hybrid protein to the cell surface (Darsow et al. 2000). The CPY-Inv overlay assay was used to identify five *L. pneumophila* proteins (VipA, VipD, VipF, 73 and LegC7) that perturb the sorting of yeast vacuolar proteins (Shohdy et al. 2005; de Felipe et al. 2008). In another study, CPY-Inv overlay assay was used to identifie three inclusion membrane proteins from *Chlamydia trachomatis*, CT105 (CteG), CT229 (CpoS) and CT223 (IPAM) which cause sorting defects in yeast (Pais et al. 2019; Bugalhão et al. 2022).

Another assay, the Ras-rescue screen is used to identify membrane-associated bacterial VFs in yeast based on the localisation of RAS GTPase to the membrane after activation by the guanine nucleotide exchange factor CDC25 (Fig. 4b) (Weigele et al. 2017). A yeast strain with a temperature-sensitive allele cdc25ts, grows normally at the permissive temperature of 25 °C but not at 37 °C. The growth defect of cdc25ts at 37 °C could be rescued by heterologous expression of nonlipidated, constitutively active Ras that is fused to a membrane-targeting domain of the protein of interest (Weigele et al. 2017). This assay was able to identify membrane-binding VFs from several Gram-negative bacteria and *Mycobacterium tuberculosis* (Weigele et al. 2017; Stamm et al. 2019). Mpt64 from *M. tuberculosis* was identified as a secreted protein for bacterial binding to eukaryotic membrane in the Ras rescue screen and it was localised in the ER during expression in yeast cells and HeLa cells. During macrophage infection, the N-terminus of Mtb bound to membrane phosphatidylinositol phosphates (PIPs), a membrane lipid which play important roles in lipid signalling, cell signalling and membrane trafficking. Mpt64 regulated macrophage response to infection by interfering with the endoplasmic reticulum (ER) with Golgi trafficking

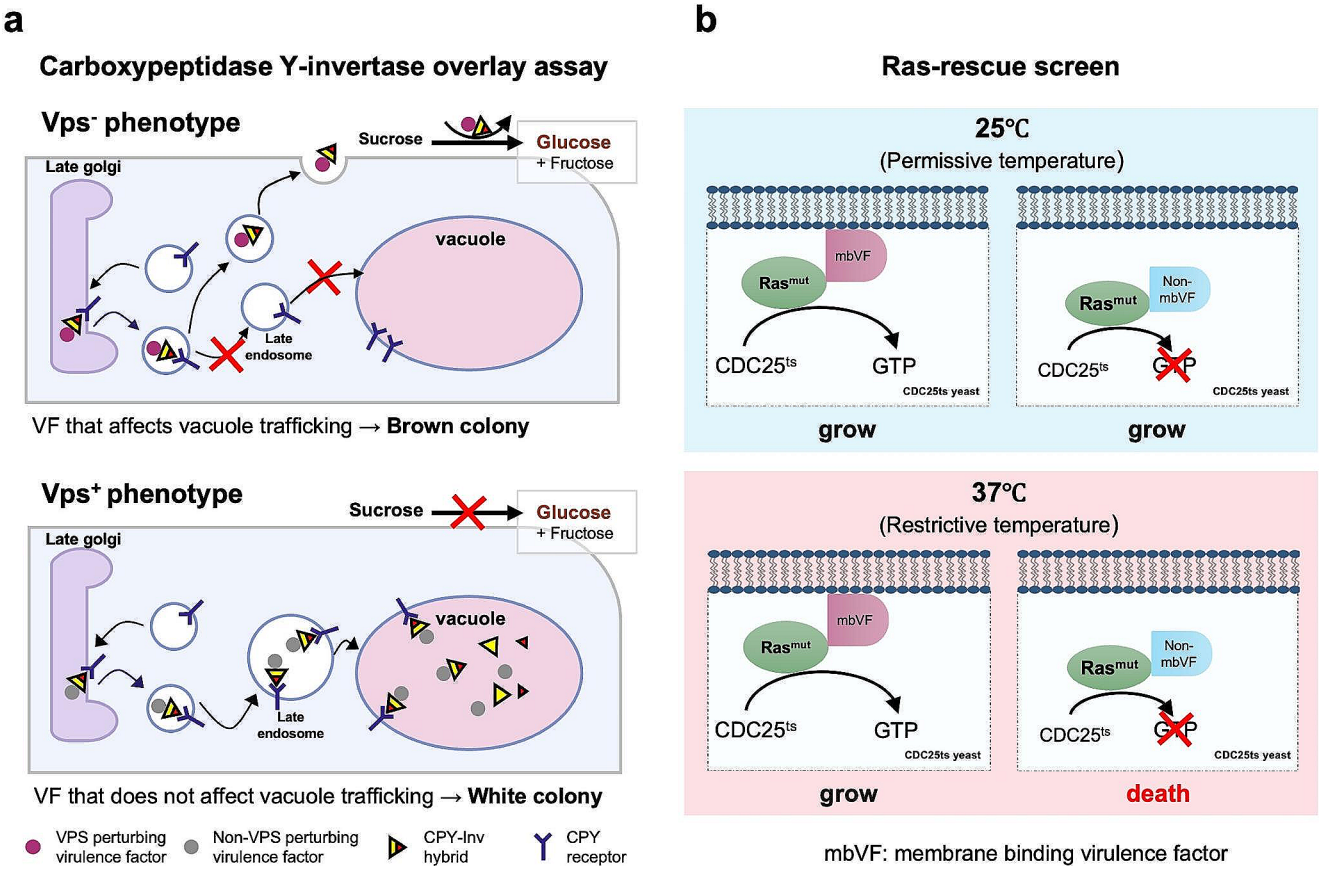

Fig. 4 Schematic diagram of (**a**) carboxypeptidase Y-invertase overlay assay that is used to screen for bacterial VFs that caused Vps defects (Vps⁻) by an assay based on the ability of the NSY01 reporter strain to produce carboxypeptidase Y-invertase (CPY-Inv), which hydrolyses sucrose to glucose and fructose on the cell surface when trafficking to the vacuole is disrupted; (**b**) Temperature-sensitive Ras-rescue screen used to identify membrane-binding bacterial VFs that can rescue yeast growth at the restrictive temperature when Ras is recruited to intracellular membranes by fusion to a membrane-binding protein in yeast

and prevented the release of the human growth hormone model substrate and inhibited the unfolded protein response (UPR) in macrophages (Stamm et al. 2019).

The observation of a synthetic growth defect in several yeast deletion mutants involved in the vesicle trafficking pathway has been used to identify bacterial VFs that are probably involved in the manipulation of ER-Golgi vesicular trafficking in yeast. For example, 12 of 26 LetA-RsmYZ-CsrA coregulated effectors of *L. pneumophila* inhibited yeast growth when overexpressed, which indicated that almost half of the LetA-RsmYZ-CsrA coregulated effectors affected conserved eukaryotic processes (Nevo et al. 2014). To identify the effectors that manipulate vesicular trafficking in yeast, all these 26 effectors were overexpressed in a *sec22*Δ mutant, which encodes an R-SNARE protein (a family of small conserved eukaryotic proteins that contribute an arginine (R) residue to mediate membrane fusion in ER-Golgi trafficking). Of the 26 effectors, 19 of them were found to be likely involved in the modulation of endoplasmic reticulum (ER)-Golgi vesicular trafficking in yeast (Nevo et al. 2014). These effectors were further examined in *sec22*Δ mutant *arf1*Δ, *arl1*Δ, and *arl3*Δ mutants, which encode small GTPases involved in the ER-Golgi trafficking and uncovered three novel effectors (i) CetLp6 which might target one of the Arf/Arl proteins; (ii) Lpg0375 which could modulate a protein of the secretory pathway that functions in this compartment; and (iii) RavH, which could target Sect. 22 or its upstream activation (Nevo et al. 2014).

Perturbing signalling cascade by bacterial virulence proteins

MAPK signalling pathway

The mitogen-activated protein kinase (MAPK) cascade is a key signalling pathway that is conserved between eukaryotic cells and regulates a variety of cellular activities, including the regulated innate immune response in mammalian cells and the response to environmental stimulation in yeast (Chen and Thorner 2007). The MAPK pathway includes three main kinases, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK, which activate and phosphorylate downstream proteins (Guo et al. 2020). Many bacterial VFs target the host MAPK signalling to manipulate host immunity and propagate infection (Nandi and Aroeti 2023). Bacterial VFs have been shown to manipulate four well-characterised MAPK signalling pathways in yeast models, including the mating pheromone pathway, the filamentous growth pathway, cell wall integrity (CWI), and the hyperosmotic growth/glycerol (HOG) pathway (Siggers and Lesser 2008; Popa et al. 2016a). Bacterial VFs can target one or multiple MAPK pathways as shown in Table 4.

These pathways have very low activity in the standard growth condition, and their activation can be triggered after response to signals or stress. The activation of MAPK pathways by stressors in growth media can increase the sensitivity of the yeast growth inhibition assay to identify bacterial virulence proteins that target thse four well-characterized cellular signalling pathways. The stressors are (i) alpha factors, which induce mating pheromone and filamentous growth pathways; (ii) heat stress, which affects general cellular metabolism, as well as the composition and structural properties of the cell wall by inducing the CWI-MAPK pathway; (iii) caffeine, has pleiotropic effects on yeast and activates the CWI-MAPK pathway; (iv) sorbitol, an osmotic stressor which creates hypotonic shock and induces both the HOG and the CWI-MAPK pathway; (v) NaCl, an osmotic and ionic stressor that induces the HOG MAPK pathway (Slagowski et al. 2008; Salomon et al. 2012; Bankapalli et al. 2017). In addition, yeast strains deleted for non-essential MAPK components of different signalling pathways were applied to determine whether yeast MAPK pathway components modulate the growth inhibition effect of bacterial VFs (Furukawa and Hohmann 2013). Stressors and yeast deletion strains have been widely involved in a variety of assays to monitor the modulation of bacterial VFs in MAPK signalling pathways, including agar plate growth phenotypic screening (Lifshitz et al. 2014; Yang et al. 2019), yeast β-galactosidase assay using the lacZ reporter or fluorescent reporter fused with the MAPK responsive gene (Salomon et al. 2012; Quaile et al. 2018), and immunoblotting with a specific anti-phospho antibody (Kramer et al. 2007; Rohde et al. 2007; Yang et al. 2019).

Here, we will describe findings on bacterial VFs that target MAPK signalling pathways in yeast. LI1035 from *L. intracellularis* can interfere with the MAPK signalling pathway by inhibiting the phosphorylation of Slt2 in yeast's CWI pathway and of the ERK pathway in mammalian cells (Yang et al. 2019). Bankapalli et al. (2017) reveal that VopE from *Vibrio cholerae* and its variant which lacks the mitochondrial target sequence, VopE$^{\Delta MTS}$ attenuates the cell wall integrity signalling pathway (CWI-MAPK) in yeast cells by activating a cellular response that opposes the function of Bck1p and Slt2p. Interestingly, co-expression of VopE$^{\Delta MTS}$ and VopX partially suppresses VopX-mediated toxicity in yeast cells (Bankapalli et al. 2017). The *Coxiella burnetti* effectors CBU0388, CBU0885, and CBU1676 target different components of the CWI-MAPK pathway. Expressions of CBU0885 and CBU1676 can increase inhibition of yeast growth in the presence of caffeine and also in yeast deletion mutants (*bck1*Δ and *mpk1*Δ) compared to the wild-type

Table 4 Summary of bacterial VFs that target MAPK signalling pathway

Virulence protein	Yeast MAPK Pathway	Plant/ Mammalian MAPK	Function in yeast	Detection method	Reference
LI1035	CWI	ERK	Inhibits the phosphorylation of Slt2.	Phenotypic screen with the presence of the stressor Immunoblotting with specific anti-phospho antibody.	(Yang et al. 2019)
Ceg4	HOG Mating pheromone	attenuates MAPK p38 activation	Attenuate phosphorylation of Hog1 and Fus3 MAP kinases.	Transcriptional reporter fusion assays immunoblotting with specific anti-phospho antibody	(Quaile et al. 2018)
VopE	CWI	-	Activate a cellular response opposing the function of Bck1p and Slt2p.	Phenotypic screen with the presence of the stressor β-Galactosidase Assay.	(Bankapalli et al. 2017)
VopX	CWI	-	Induce signalling through Rlm1, resulting in growth inhibition and the activation of Rlm1 responsive promoters.	β-Galactosidase assay Transcriptional reporter fusion assays	(Seward et al. 2015)
CBU0388	CWI	-	Enhances the activation of yeast CWI-MAPK pathway.	Phenotypic screen with the presence of the stressor	(Lifshitz et al. 2014)
CBU0885 CBU1676			Inhibit activation of yeast CWI-MAPK pathway .	β-Galactosidase Assay (transcriptional reporter fusion assays)	
NopM	Mating pheromone	-	Inhibit STE4-induced mating pheromone signalling.	Pheromone sensitivity halo assays Immunoblotting with specific anti-phospho antibody	(Xin et al. 2012)
AptA	CWI	Erk1/2 (MEK1/2)	Inducing phosphorylation of Mpk1 and activation of Rlm1.	Immunoblotting with specific anti-phospho antibody β-Galactosidase Assay (transcriptional reporter fusion assays)	(Sukumaran et al. 2011)
HopX1	HOG	-	Specifically attenuated the activation of the high osmolarity glycerol (HOG) mitogen-activated is dependent on the putative transglutaminase catalytic triad of the effector, without affecting Hog1 expression level or nuclear entry dynamics.	Phenotypic screen with the presence of the stressor Pheromone sensitivity halo assays β-Galactosidase Assay	(Salomon et al. 2012)
VopX	CWI	-	Stimulating the CWI pathway through Rlm1	Phenotypic screen with the presence of the stressor β-Galactosidase Assay	(Alam et al. 2011)
NopL	Mating pheromone	Suppressed cell death induced either by overexpression of the MAPK gene SIPK (salicylic acid-induced protein kinase) or by SIPK(DD) (mutation in the TXY motif resulting in constitutive MAPK activity)	Disrupted the mating pheromone (α-factor) response pathway	Phenotypic screen with the presence of the stressor Pheromone sensitivity halo assays	(Zhang et al. 2011)
IpaH9.8	Mating pheromone	-	Inhibits yeast pheromone-induced MAPK pathway by promoting the proteasome-dependent destruction of the MAPKK Ste7	Phenotypic screen with the presence of the stressor Pheromone sensitivity halo assays immunoblotting with specific anti-phospho antibody	(Rohde et al. 2007)

Table 4 (continued)

Virulence protein	Yeast MAPK Pathway	Plant/ Mammalian MAPK	Function in yeast	Detection method	Reference
OspF	CWI	MAPK phosphatase for ERK and p38	The OspF-dependent reversed regulation of CWPI, PRM5, and FIT2 suggested that OspF expression directly or indirectly inhibits RLM1-regulated transcription. Inhibit activation of the CWI pathway. OspF targets a protein upstream of RLM1 and downstream of RHO1 in the CWI pathway.	β-Galactosidase Assay Immunoblotting with specific anti-phospho antibody	(Kramer et al. 2007)
VopA	HOG	-	Inhibit activation of the yeast MAPKs Hog1p and Mpk1p	Phenotypic screen with the presence of the stressor immunoblotting with specific anti-phospho antibody	(Trosky et al. 2004)
YopJ	HOG Mating pheromone	-	Blocking phosphorylation of MAPKK Ste7p and Pbs2p	Phenotypic screen with the presence of the stressor Pheromone sensitivity halo assays β-Galactosidase Assay	(Yoon et al. 2003)

strain, while CBU0388 shows the opposite effect. CBU0388 enhanced the activation of the yeast CWI-MAPK pathway, while CBU0885 and CBU1676 inhibited this activation. Furthermore, CBU1676 and CBU0388 could oppositely affect the same target, since they suppress the effect of each other on yeast growth (Lifshitz et al. 2014). Ceg4 from *L. pneumophila* is a phosphotyrosine phosphatase that attenuates the activation of eukaryotic MAPK pathways. Ceg4 has been shown to attenuate the phosphorylation of Hog1 and Fus3 MAP kinases in yeast while attenuating MAPK p38 activation in mammalian cells (Quaile et al. 2018).

However, the yeast model may not accurately reflect the activity of bacterial VFs in the MAPK pathway and their homologous target in the host cell. The Shigella effector IpaH9.8, an E3 ubiquitin ligase, targeted MAPKK Ste7 in yeast but not its closest mammalian homologues (Rohde et al. 2007). The *V. cholerae* VopE and XopE2 did not affect the activation of the lacZ-MAPK-responsive reporter for the yeast HOG pathway and the CWI pathway respectively, but exhibited growth inhibition effect in the presence of their corresponding stressors, NaCl and sorbitol (HOG pathway) in the case of VopE, and caffeine (CWI pathway) in the case of XopE (Bosis et al. 2011; Bankapalli et al. 2017).

TORC1 signalling pathway

Target of rapamycin complex 1 (TORC1) is an evolutionarily conserved Ser/Thr-protein kinase and plays an important role in coordinating cell growth and metabolism in response to nutrients and growth factors (Morozumi and Shiozaki 2021). The TORC1 complex contains Tor1 or Tor2 protein kinases and can be inhibited by the drug rapamycin. The yeast model has been used to identify the bacterial VFs that target the TORC1 signalling pathway and their host cofactors based on sensitivity to rapamycin. For example, the expression of *Ralstonia solanacearum awr5* inhibited yeast growth but did not cause cell death. A genome-wide transcriptomic analysis using DNA microarrays in yeast cells with AWR5 expression showed that its transcriptomic profile changes are similar to TOR inhibition by rapamycin or nitrogen starvation (Popa et al. 2016b). Mutations in yeast *cdc55* and *tpd3*, which encode regulatory subunits of protein phosphatase 2 A in TORC1-regulated pathways, were able to suppress AWR5-induced growth inhibition in yeast (Popa et al. 2016b). These suggest that AWR5 impacts TORC1-regulated pathways in eukaryotic cells. Furthermore, the expression of *Shigella* cysteine protease, OspB in yeast was hypersensitised to the presence of rapamycin, an inhibitor of the TORC1 signalling pathway due to the cleavage of the TORC1 component Tco89p (Wood et al. 2022). Using a PGA screen, inositol hexakisphosphate was identified as one of the host factors required for OspB-induced growth inhibition.

Rho GTPase signalling cascades

Rho GTPases are a subfamily of the Ras superfamily of small GTP-binding proteins, and they switch between active GTP-bound and inactive GDP-bound states to regulate signal transduction pathways in eukaryotic cells by GAP (GTPase-activating protein) and GEF (guanine-nucleotide-exchange factor) respectively (Mosaddeghzadeh and Ahmadian 2021). Rho GTPase is highly conserved between yeast and humans, and functions to control actin dynamics, vesicle trafficking, cell cycle, and migration (Mosaddeghzadeh and Ahmadian 2021; Eckenstaler et al. 2022). Several

bacterial VFs target eukaryotic cells by modulating the Rho GTPase signalling cascade to promote invasion and proliferation within their host or to help bacteria escape immune defences and phagocytosis (Popoff 2014; Chaoprasid and Dersch 2021). Several Rho GTPase targeting VFs have been studied in yeast model, as shown in Table 5.

Manipulation of ADP-ribosyltransferase activities

Bacterial ADP-ribosyltransferase (ART) toxin family is a group of bacterial toxins that removes the ADP-ribose group from NAD^+ and covalently binds to a variety of eukaryotic targets such as Rho proteins, heterotrimeric G proteins and actin (Simon et al. 2014; Groslambert et al. 2021; Chaoprasid and Dersch 2021). These ART toxins will inhibit or modify normal eukaryotic protein function to promote bacterial pathogenesis and lead to cell death. The yeast heterologous expression system had been used to identify and characterise the bacterial ART toxins such as diphtheria toxin 2subgroup (ExoA, DT and cholix) and C3 toxin subgroup from *Paenibacillus larvae* (Plx2A, C3larvin and C3larvinA) which target RhoA by revealing their catalytic residue/domain for enzymatic activity in yeast cells (Arnoldo et al. 2008; Turgeon et al. 2009; Krska et al. 2015; Ebeling et al. 2017; Turner et al. 2020). The yeast-based assay also revealed that the mutant of the target protein of elongation factor 2 in yeast, G701R, confers resistance to all toxins in the DT group and rescues the yeast from growth defect (Turgeon et al. 2009). Using the yeast ORF deletion and overexpressing library, Arnoldo et al. demonstrated that overexpression of Ras2p, a homologue of the human Ras protein and deletion of Bmh1p, Brain Modulosignalin Homolog in yeast can suppress the growth inhibition effect from overexpression of *Pseudomonas aeruginosa* ART toxin, ExoU (Arnoldo et al. 2008). Later on, an in vitro enzymatic assay was performed and revealed that Ras2p was directly ADP-ribosylated by ExoS with Bmh1p as a cofactor (Arnoldo et al. 2008).

Table 5 Summary of bacterial VFs that target Rho GTPase signalling pathway

Virulence protein	Function in yeast	Approaches involved yeast	Reference
SteC	Alters the actin cytoskeleton by directly inhibiting Cdc42-mediated signalling by binding to Cdc24, the sole GEF controlling Cdc42	Yeast multicopy suppression screen Fluorescence microscopy Yeast two-hybrid assay	(A. Alemán 2009; Fernandez-Piñar et al. 2012)
YopT	Act as a cysteine protease that cause proteolytically inactivation on post-translationally modified Rho family GTPases, including RhoA, Rac and Cdc42	Yeast multicopy suppression screen	(Shao et al. 2002)
YopE	Function as GAP for Rho GTPases, inhibited the polarization of the yeast cytoskeleton and resulted in the inhibition of yeast bud formation	Yeast multicopy suppression screen	(Von Pawel-Rammingen et al. 2000)
IpgB2	Functions as a GEF to specifically subvert the Rho1 signalling pathway	PGA screen DNA microarray mRNA profiling	(Alto et al. 2006)
CirA	Target Rho1 in yeast to cause yeast growth inhibition	Yeast multicopy suppression screen	(Weber et al. 2013, 2016)

Modulation of programmed cell deaths by bacterial virulence proteins

Apoptosis is a process of programmed cell death (PCD) that is required for the development and homeostasis of multicellular organisms through intracellular breakdown of harmful or damaged cells and their engulfment by phagocytic cells (Bedoui et al. 2020). Apoptosis is triggered by either an intrinsic (mitochondrial) or extrinsic (cell surface receptors) signalling pathway that leads to organelle dysfunction and activation of the caspase-signalling cascade (Bedoui et al. 2020). Modulation of apoptosis in phagocytic cells becomes one of the strategies used by bacterial pathogens to overcome host defence systems (Wanford et al. 2022). Bacterial pathogens can induce apoptosis for bacterial invasion, dissemination, and by killing macrophages with pore-forming toxins, protein synthesis inhibitors, or exotoxins (Selvaraj et al. 2021). Conversely, some bacterial pathogens can also inhibit apoptosis, preventing the engulfment of apoptotic-infected cells to evade innate host defences (Behar and Briken 2019).

Yeast is an established model organism to study apoptosis of higher eukaryotes, since the apoptotic core machinery is conserved in yeast and several yeast orthologues of crucial mammalian apoptotic proteins have been identified (Manon 2022). The apoptosis phenotypes in yeast that are triggered by the expression of bacterial VFs have been studied with the aid of yeast deletion strains related to the apoptotic pathway, and apoptotic cell death assays using DAPI and transmission electron microscopy (TEM) to observe apoptosis phenotypes in yeast cells; additionally Annexin V and propidium iodide (PI) staining to detect phosphatidylserine externalisation; and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay to detect DNA fragmentation (Deng et al. 2016). The combination of these approaches revealed that *R. solanacearum* RipI exhibits apoptosis phenotypes in yeast cells that are independent of the hydrogen peroxide-mediated apoptosis pathway

and mitochondrial-mediated apoptotic pathways. RipI was observed to be localised in the yeast nucleus and triggers DNA damage-related apoptosis dependent on its integrase function (Deng et al. 2016).

In addition to that, the yeast model can serve as a powerful tool for the functional study of proteins involved in apoptosis, such as members of the Bcl-2 family, a group of apoptosis regulators (Manon 2022). The Bax protein is one member of the Bcl-2 family and the key regulator of the intrinsic pathway of apoptosis (Peña-Blanco and García-Sáez 2018). The expression of mammalian pro-apoptotic Bax in yeast induced cell death by causing loss of mitochondrial outer membrane potential, cytochrome c release, and promoted plasma membrane integrity, which are the same characteristics shared with higher eukaryotes (Khoury and Greenwood 2008). The Bax-induced cell death in yeast can be reverted by co-expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L and Mcl-1 (Xu et al. 2000). Using this approach, the *Brucella melitensis* porin Omp2b was identified as a suppressor of Bax-induced cell death through the screening of a yeast library expressing *B. melitensis* ORFs (Laloux et al. 2010).

Belyi et al. (2012) used budding yeast to study the toxic activity of *Legionella* glucosyltransferase, Lgt1, which is a toxin that causes post-translational modification of host proteins by sugar attachment (Jank et al. 2015). Lgt1 has been shown to inhibit in vitro protein synthesis and induce cell death in mammalian and yeast cells by modifying serine53 in mammalian elongation factor 1 A (eEF1A), which is responsible for the enzyme delivery of aminoacyl tRNAs to the ribosome and its yeast analogue elongation factors, Tef1 (Belyi et al. 2006, 2012). The yeast mutant, TEF1-Ser53Ala, could not be glycosylated by Lgt1 and was resistant to Lgt1 toxicity. However, the deletion of the Hbs1 gene in yeast, another substrate of *Legionella* glucosylating enzymes, did not influence the toxic effects caused by Lgt1.

Several pathogenic bacteria such as *Campylobacter* spp., *E. coli, and Shigella dysenteriae* secrete a cytolethal distending toxin (Cdt), a heat-labile genotoxin that causes DNA damage in target cells and acts as a triperditious toxin that affects host defences, leading to cell cycle arrest and cell death via apoptosis (Pons et al. 2019; Kailoo et al. 2021). Cdts are AB_2 heterotrimeric holotoxins and are composed of three subunits: CdtB (functions as a PIP_3 phosphatase and DNAase I), CdtA and CdtC (interact with the membrane and deliver CdtB from the membrane to the nucleus) (Kailoo et al. 2021). In 2001, Hassane et al. (2001) established a yeast model to study the in vivo mechanism of Cdt toxicity. A combination of phenotypic analysis on yeast and its mutant and flow cytometry analysis of DNA content demonstrated that the expression of CdtB alone was sufficient to induce irreversible G_2/M cell cycle arrest by phosphoesterase activity in yeast as well as in mammalian cells. Next, Kitagawa et al. (2007) established a genome-wide screen in the yeast deletion library on the Cdt subunit, CdtB. There are 61 CdtB-sensitive deletion strains involved in the component of DNA metabolism, chromosome segregation, vesicular traffic, RNA catabolism, protein translation, morphogenesis, or nuclear transport, as well as an unknown open reading frame. These selected mutants were further tested for their sensitivity to homothallic switching endonuclease, which is a direct DNA double-strand break (DSB), indicating that CdtB-induced DNA damage is not similar to direct DSB as not all of them are sensitive. To elucidate the molecular pathway involved in the function of CdtB, CdtB from *A. actinomycetemcomitans*, AaCdtB was expressed in a yeast and found to cause DNA damage, S/G2 cell cycle arrest, and cell damage due to its DNAase I activities (Matangkasombut et al. 2010).The hypersensitivity screen of yeast deletion strains to AaCdtB reveals that yeast strains with defects in homologous recombination (HR) repair, but not other repair pathways, are hypersensitive to AaCdtB. Besides that, the effect of AaCdtB in yeast strains with mutations in apoptotic regulators was examined and showed that yeast death occurrence was partially dependent on histone H2B serine 10 phosphorylation but not dependent on yeast metacaspase gene, *YCA1* and the apoptosis-inducing factor, *AIF1* (Matangkasombut et al. 2010). Therefore, the host factors required for AaCdtB translocation and cytotoxicity were identified in the genome-wide screen for mutations that confer AaCdtB resistance (Denmongkholchai et al. 2019).

Conclusion

In this review, we discussed the tools, principles and applications of yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as model organisms to identify and characterise bacterial VFs that perturbed conserved cellular processes among eukaryotes. Many approaches using yeast from small-scale to genome-wide analysis have been developed from the implementation of the growth inhibition phenotypes caused by overexpression of bacterial VFs. The yeast model not only reveals the interaction between bacterial VFs and targeted host molecules, but also the regulation of other cellular processes during pathogenesis. However, it is important to remember that there are differences between the native host (humans, animals, plants) and the yeast cell. Yeast lacks an immune system and some of the specific host-pathogen interactions that occur during bacterial infections cannot be fully replicated in yeast. Therefore, yeast models are used alongside other model systems, such as cell lines or animal models, for a more comprehensive understanding of

bacterial virulence. Yeast can provide initial insights, which can then be further validated in more complex models. The limitations can be overcome by developing yeast strains with engineered features that better mimic mammalian host cells in the future. Hence, the yeast model system is likely to remain a valuable tool, especially for studying single bacterial VFs, to help researchers identify potential drug targets or develop strategies to counteract bacterial pathogenesis, with the integration of other approaches.

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Declarations

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

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