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LI1035, a putative effector secreted by *Lawsonia intracellularis*, targets the MAPK pathway and regulates actin organizationin yeast and mammalian cells

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ABSTRACT

Lawsonia intracellularis is an obligate intracellular Gram-negative bacterium that has been identified as the etiological agent of the contagious disease proliferative enteropathy (PE) in a wide range of animals, mainly pigs. The genome sequence of *L. intracellularis* indicates that this bacterium possess a type III secretion system (T3SS), which may assist the bacterium during cell invasion and host innate immune system evasion and could be a mechanism for inducing cellular proliferation. However, the effectors secreted by the T3SS (T3Es) of *L. intracellularis* have not been reported. T3Es often target conserved eukaryotic cellular processes, and yeast is an established and robust model system in which to reveal their function. By screening the growth inhibition of an ordered array of Saccharomyces cerevisiae strains expressing the hypothetical genes of L. intracellularis, LI1035 was identified as the first putative effector that inhibits yeast growth. The LI1035-induced growth inhibition was rescued in two of the 14 mitogen-activated protein kinase (MAPK) yeast haploid deletion strains, suggesting that LI1035 interacts with the components of the MAPK pathway in yeast. Phosphorylation assays confirmed that LI1035 inhibits MAPK signaling cascades in yeast and mammalian cells. Actin staining assays revealed that LI1035 regulates actin organization in yeast and mammalian cells. Taken together, these results indicate that LI1035 alters MAPK pathway activity and regulates actin organization in the host. These findings may contribute to the understanding the pathogenesis of *L. intracellularis* and support the use of yeast as a heterologous system for the functional analysis of pathogen-specific gene products in the laboratory.

1. Introduction

Lawsonia intracellularis is a novelg Gram-negative bacterium and the etiological agent of a commercially significant disease—proliferative enteropathy (PE)—in numerous wild and domestic animal species, most notably pigs and horses (Karuppannan and Opriessnig, 2018; Vannucci and Gebhart, 2014). The infection is characterized by proliferation, hemorrhage, necrosis, or any combination commonly referred to as “ileitis” and affects the health and production efficacy of farmed pigs (Karuppannan and Opriessnig, 2018; Vannucci and Gebhart, 2014). Currently, the understanding of *L. intracellularis* pathogenesis and host–pathogen interactions is limited due to this organism’s fastidious microaerophilic obligate intracellular nature, genetic intractability and by the inability to reproduce cellular proliferation, the main phenotypic characteristic of *L. intracellularis* infection, *in vitro* (Karuppannan and Opriessnig, 2018; Vannucci and Gebhart, 2014).

The *L. intracellularis* genome was first sequenced in 2003. Analysis of the genomic sequence of *L. intracellularis* revealed the presence of a type III secretion system (T3SS) (Alberdi et al., 2009). This secretion apparatus exists in many other gram-negative bacterial pathogens of plants and animals and translocates a suite of effector proteins (Type III-secreted effectors, T3Es) directly into the cytosol of host cells, where they act as virulence factors modulating various cellular processes and suppressing host defense responses for the benefit of the pathogen (Barison et al., 2013; Popa et al., 2016). Identification and characterization of T3Es are vital but particularly complex undertakings, as the amino acid sequences of most effectors are not significant similar to those of proteins of known function and contain no easily recognizable secretion signals (Popa et al., 2016). Indeed, to date, the effectors secreted by the *L. intracellularis* T3SS have not been reported.

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As a heterologous surrogate host, the budding yeast *Saccharomyces cerevisiae* is an established and robust model system for the identification and functional characterization of T3Es (Popa et al., 2016; Siggers and Lesser, 2008). When expressed in *S. cerevisiae*, T3Es often target conserved cellular processes and induce robust phenotypes, such as growth inhibition (Siggers and Lesser, 2008). In contrast, the expression of very few non-translocated bacterial proteins (less than 1%) affects yeast growth (Liu et al., 1992). In numerous studies on effectors produced by bacterial pathogens of plants and animals, growth inhibition phenotypes have been exploited to elucidate the functions and identify the targets of the effectors (Siggers and Lesser, 2008).

Mitogen-activated protein kinase (MAPK) signaling pathways have been studied in great detail in *S. cerevisiae* and mammalian cells (Chen and Thorner, 2007; Morrison, 2012) and have been shown to be targeted by several T3Es produced by diverse pathogenic bacteria (Popa et al., 2016; Shan et al., 2007; Siggers and Lesser, 2008). For instance, inhibition of MAPK phosphorylation is a common effect of the interaction of T3Es with the eukaryotic cell signaling machinery (Kramer et al., 2007).

Actin is a well-known molecule whose function of dynamic filament assembly has been appropriated into numerous cellular processes in prokaryotes and eukaryotes (Smethurst et al., 2014). Subversion of the host actin cytoskeleton is a critical virulence mechanism used by various bacterial pathogens during their infectious life cycle (Popa et al., 2016; Siggers and Lesser, 2008). Bacterial pathogens reorganize the actin cytoskeleton to promote their uptake into the host cell or prevent their phagocytosis by macrophages (de Souza Santos and Orth, 2015; Lamason and Welch, 2017).

To circumvent limitations encountered in the investigation of effector functions and expedite the discovery of *L. intracellularis* targets, we screened the effects of a repertoire of hypothetical genes on growth inhibition in the budding yeast *S. cerevisiae* and identified LI1035 as the first *L. intracellularis* effector protein that inhibits yeast growth. We showed that LI1035 can interfere with the MAPK signaling pathway and regulate actin organization when ectopically expressed in yeast and mammalian cells. Our study strongly supports the hypotheses that LI1035 targets conserved cellular processes in yeast and mammalian cells and that yeast will be instrumental in elucidating the functions and roles of *L. intracellularis* effectorsin pathogenesis.

2. Materials and methods

2.1. Medium, strains and cell line

The bacterial and yeast strains used in this study are described in Table 1. The E. coli strain DH5α was used for general cloning and propagated in Luria-Bertani (LB) broth supplemented with 100 μg ml^{-1} Ampicillin or 50 μg ml^{-1} Kanamycin at 37°C with agitation or on LB agar plates. The template genomic DNA from the *L. intracellularis*-positive porcine ileal mucosal DNA was exact as the manufacturer's instructions. The PCR products were analyzed by 1.2% agarose gel electrophoresis and purified by a QIA quick DNA gel extraction kit (Qiagen, Switzerland). The restriction enzymes were offered by TaKaRa Biotech Co., Ltd., Japan.

Table 1
Strains used in this study.

Strains	Genotype
DH5α	F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK +) phoA supE44 λ– thi-1 gyrA96 relA1
W303-1A	MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100
BY4741	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0
BY4741 Δste20	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; ste20Δ::kanMX4
BY4741 Δste11	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; ste11Δ::kanMX4
BY4741 Δste7	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; ste7Δ::kanMX4
BY4741 Δfus3	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; fus3Δ::kanMX4
BY4741 Δkss1	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; kss1Δ::kanMX4
BY4741 Δsho1	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; sho1Δ::kanMX4
BY4741 Δpbs2	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; pbs2Δ::kanMX4
BY4741 Δhog1	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; hog1Δ::kanMX4
BY4741 Δbck1	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; bck1Δ::kanMX4
BY4741 Δslt2	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; slt2Δ::kanMX4
BY4741 Δrlm1	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; rlm1Δ::kanMX4
BY4741 Δrho2	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; rho2Δ::kanMX4
BY4741 Δrho4	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; rho4Δ::kanMX4
BY4741 Δrho5	MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; rho5Δ::kanMX4

The yeast strains W303-1A and BY4741 were grown at 30°C in yeast-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) broth or agar (2%) or in selective synthetic complete (SC) medium lacking uracil (SC-Ura-) or lacking both uracil and leucine (SC-Ura- Leu-) to maintain the plasmid and supplemented with 2% glucose (SCD), or 2% galactose and 1% raffinose (SCG) as carbon sources. The components for the media were purchased from HiMedia and Difco. All BY4741 deletion strains were purchased from Thermo Fisher (95401.H3), verified by UP TAG (barcode) confirmation and grown at 30°C in YPD, SC-Ura-, or SC-Ura-Leu- plate supplemented with 200 μg ml^{-1} G418.

The human embryonic kidney (HEK) 293 T cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone), 1 mmol L^{-1} glutamine, and 100 U ml^{-1} each of penicillin and streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Plasmid construction

The plasmids used in this study are described in Table 2. The yeast expression vector pRS416-GAL1 was constructed by inserting a synthesized double-stranded DNA fragment encoding the GAL1 promoter, Flag-tag and the CYC1 terminator into pRS416 using the restriction enzymes Sac I and Kpn I.

The primers used to amplify hypothetical genes of L. intracellularis were synthesized by Sangon Biotech (Shanghai) Co., Ltd., China and listed in Table S1. The hypothetical ORF of L. intracellularis was cloned into pRS416-GAL1 by homologous recombination as described previously (Sisko et al., 2006), and the sequences of the oligonucleotides used to amplify these hypothetical genes are listed in Table S1 and are available in the online version of this paper. Briefly, oligonucleotide primers (59 nt.) with a 5′-terminus complementary to the sequence of the galactose-inducible yeast expression vector pRS416-GAL1 and a 3'-terminus specific for the gene of interest were used to amplify the ORF from *L. intracellularis*-positive porcine ileal mucosal DNA by polymerase chain reaction (PCR). Each PCR product was transformed into yeast with linearized pRS416-GAL1 plasmid (digested by NdeI and XhoI). The two DNA molecules were efficiently spliced by homologous recombination to generate a replication-competent plasmid and an in-frame gene fusion of the Flag-tag and L. intracellularis ORF. Plasmids were recovered from *Ura*$^{+}$ transformants and sequenced to confirm correct splicing.

The primers used in this study are shown in Table 3. The high-copy number yeast expression vectors pESC-LEU-KSS1 and pESC-LEU-SLT2 were constructed by inserting the coding sequences of KSS1 and SLT2 from DNA extracted from the *S. cerevisiae*W303-1A strain into pESC-LEU using the restriction enzymes *BamH* I and *Sac*I respectively. The mammalian expression vector pcDNA3.1-LI1035 was constructed by inserting a synthesized double-stranded DNA fragment encoding the Flag-tag and 1029 bp of the LI1035 sequence into pcDNA3.1 (-) by using the restriction enzymes Not I and BamH I. The mammalian expression vector pcDNA3.1-EGFP-LI1035 was constructed by inserting the fusion PCR product of EGFP and LI1035 into pcDNA3.1 by using the restriction enzyme *BamH I*

Table 2
Plasmids used in this study.

plasmids	Genotype/Description	source
pYES2/NTA	2μ origin, GAL1 promoter, URA3, ampicillin	Invitrogen
pYES2/NTA-RiPI	RipI gene fragment amplified from genomic DNA of Ralstonia solanacearum strain GMI1000 by PCR and cloned into pYES2/NTA under GAL1 promoter	Deng MY et al, 2016
pRS416	URA3, ampicillin	
pRS416-GAL1	GAL1 promoter, Flag tag, URA3, ampicillin	This study
pRS416-GAL1-LI1035	1029 bp LI1035 gene fragment amplified from genomic DNA of *L. intracellularis* positive porcine ileal mucosal was cloned into pRS416-GAL1 by homologous recombination under GAL1 promoter.	This study
pESC-LEU	2μ origin, GAL1/GAL10 divergent promoters, LEU2, ampicillin	Agilent
pESC-LEU-KSS1	1107 bp KSS1 gene fragment amplified from genomic DNA of BY4741 with BamHI/SalI primers by PCR and cloned into pESC-LEU under GAL1 promoter	This study
pESC-LEU-SLT2	1455 bp SLT2 gene fragment amplified from genomic DNA of BY4741 with *BamHI/SalI* primers by PCR and cloned into pESC-LEU under GAL1 promoter	This study
pCDNA3.1(-)	CMV, Neomycin, ampicillin	Invitrogen
pCDNA3.1-LI1035	synthesized double-stranded DNA fragment encoding Flag-tag and 1029 bp of LI1035 was inserted into pCDNA3.1(-)using the restriction enzymes Not I and BamH I	This study
pCDNA3.1(+)	CMV, Neomycin, ampicillin	Invitrogen
pEGFP-N1	CMV, neomycin, kanamycin, EGFP	Clontech
pCDNA3.1-EGFP	EGFP gene fragment amplified from plasmid by pEGFP-N1 and cloned into pYES2/NTA under GAL1 promoter	He et al., 2019
pCDNA3.1-LI1035-EGFP	The fusion PCR product of LI1035 and EGFP with *BamH* I primers was cloned into similarly digested pCDNA3.1	This study

2.3. Yeast growth assays

pRS416-GAL1 expression vectors carrying *L. intracellularis* genes were transformed into the *S. cerevisiae* strains W303-1A and BY4741 by the lithium acetate transformation method. Transformed yeast cells were plated onto SCD. Yeast growth assays were performed as described previously (Salomon et al., 2011). Briefly, overnight cultures of all recombinant strains grown in SCD-Ura- medium were washed, and absorbance values were normalized to an optical density at 600 nm (OD_{600}) of 1.0. Each strain was 10-fold serially diluted four times and spotted (4 μl) onto repressing (2% glucose) or inducing (2% galactose) solid selective medium or onto inducing medium supplemented with sorbitol (0.8 M).Yeast cultures were then incubated at 20 °C, 30 °C or 37 °C for 2–4 days.

2.4. Preparation of yeast extracts and immunoblot analysis

To verify the expression of LI1035 or RipI in yeast, immunoblot analysis was carried out. Briefly, the recombinant yeast strains were grown in SCD-Ura- medium at 30 °Cto exponential phase (OD_{600}˜0.8–1.0). Cells were collected, washed and diluted in selective induction medium (2% galactose). After 12 h of induction, cells were pelleted, and whole-cell protein extracts were prepared. Equal amounts of protein samples were fractionated by SDS-PAGE using 12% gels and transferred to PVDF membranes (Millipore). Membranes were probed with mouse anti-Flag or anti-Xpress antibodies (Invitrogen). The primary antibody was detected using a horseradish peroxidase conjugated anti-mouse IgG antibody, and the blot was developed with Super Signal chemiluminescence kits (Pierce).

2.5. Yeast MAPK phosphorylation assays

Yeast MAPK phosphorylation assays were performed as previously described (Kramer et al., 2007). Briefly, yeast transformed with pRS416-GAL1-LI1035 or pRS416-GAL1 were grown overnight in SC-Ura- medium supplemented with 2% raffinose. Cultures were then diluted to OD_{600} = 0.2–0.3 in fresh medium and incubated for 2 h at 30°C. LI1035 expression was induced by the addition of 2% galactose. Cells were incubated for 2 h at 30 °C before exposure to the stresses. Cells were shocked by 1:1 dilution with medium pre-warmed to 55°C followed by incubation at 39°C for 30 min. The shock response was terminated by an additional 1:1 dilution with ice-cold stop mix. The mating and invasive growth MAPK pathways were induced by the addition of 200 nM a-factor for 15 min. The high-osmolarity glycerol (HOG) pathway was induced by the addition of 400 mM NaCl for 5 min. In all cases, yeast cells were pelleted and snap-frozen at the completion of the shock procedure. Protein was isolated from yeast cells and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes, which were probed with the indicated antibodies. The phospho-p42/44 antibody was used to recognize yeast phosphorylated SLT2, FUS3 and KSS1. The phospho-p38 antibody was used to recognize yeast phosphorylated HOG1. The GAPDH antibody was used as the loading control. The phospho-p42/44 antibody, phospho-p38 antibody and GAPDH antibody were purchased from Santa Cruz Biotechnology.

Table 3
Primers used in this study.

Primer	Sequence (Restriction enzyme sites are underlined)	Restriction enzyme
pESC-LEU-KSS1-F	CGGGATCCATGGCTAGAACCATAACTT	*BamH*I
pESC-LEU-KSS1-R	GCGTCGACCTATTCCATGGTCTTCATTAGTTCA	*Sal*I
pESC-LEU-SLT2-F	CGGGATCCATGGCTGATAAGATAGAGAG	*BamH*I
pESC-LEU-SLT2-R	GCGTCGACAAAATATTTTCTATCTAATC	*Sal*I
pCDNA3.1(-)-LI1035-F	GACGATGACAAGCTTGCGGCCGCGTAGACTGTTCTATAAAAAAT	*Not*I
pCDNA3.1(-)-LI1035-R	GAACCATGGCTCGAGGGATCCTTAAGTATGTTGTGTTTCTAAAG	*BamH*I
pCDNA3.1(+)-LI1035-EGFP-F*[1]	CTTGGTACCGAGCTCGGATCCGTAGACTGTTCTATAAAAAATAAGAAAGG	*BamH*I
pCDNA3.1(+)-LI1035-EGFP-R*[2]	**ACCTCCGCTACCTGTATCAGCTCC**TTAAGTATGTTGTGTTTCTAAAGTATC	
EGFP-F*[2]	**GGAGCTGATACAGGTAGCGGAGGT**ATGGTGAGCAAGGGC	
EGFP-R*[1]	CTTGGTACCGAGCTCGGATCCTTACTTGTACAGCTCGTCC	*BamH*I

*[1]The primers used for fusion PCR.
*[2]The overlap sequence of fusion PCR. The spacer sequence that codes for peptide 'GADTGSGG' which can facilitate the correct folding of the fluorescent proteins when coupled to the protein of LI1035.

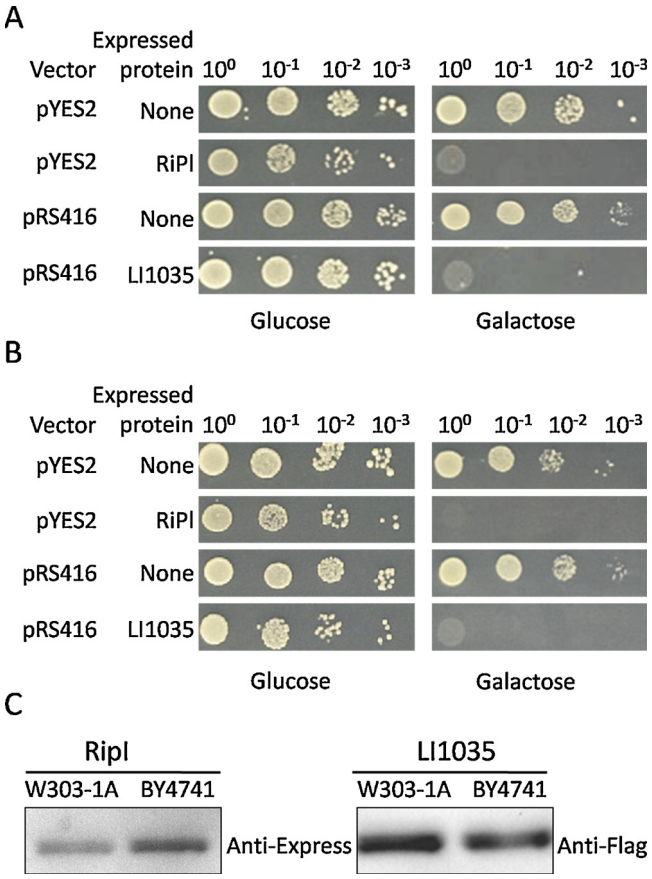

Fig. 1. Yeast growth is inhibited by *L. intracellularis* LI1035 expression. (A)W303-1A yeast cells harboring the yeast expression vector pRS416-GAL1 (a low copy number vector), either empty or encoding LI1035, were grown overnight in repressing medium (2% glucose).Culture absorbance values were then normalized to an OD_{600}~1.0, and serial 10-fold dilutions were spotted onto repressing or inducing medium (2% galactose).Cells expressing RipI in the pYES2/NT vector were the positive control for growth inhibition. Images were obtained after 2 and 3 days of growth at 30°C for yeast growing in repressing and inducing medium, respectively. The data shown are representative of the results of three independent experiments. (B) A similar experiment was performed in the BY4741 yeast strain. (C)Induction of expression was verified by Western blotting using an anti-Xpress antibody for pYES2/NT-RipI and an anti-Flag antibody for pRS416-LI1035.

2.6. Mammalian MAPK assays

Asynchronously growing HEK293 T cells were seeded at 2.5×10^5 cells/well in a 6-well tissue culture plate and serum-starved overnight. Two micrograms of plasmid DNA was transfected into HEK293 T cells with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. The 6-well plates were subsequently incubated at 37°C for 24 h, and 50 ng ml^{-1}epidermal growth factor (EGF) was added for 30 min. Cells were washed with ice-cold PBS containing1 mM Na3VO4 and 10 mM NaF and were then lysed with 300 µl of RIPA buffer containing protease inhibitors. Equal volumes of samples were subjected to SDS-PAGE. Proteins were transferred to PVDF membranes, which were probed with the phospho-p42/44 antibody, p42/44 antibody (Santa Cruz) or GAPDH antibody.

2.7. Yeast actin staining

Yeast cells were grown in 2% raffinose to logarithmic phase and induced to express LI1035 by the addition of 2% galactose for 5 h. Cells were fixed in 3.7% formaldehyde for 30 min at room temperature, washed twice with PBS containing 1 mg ml^{-1} BSA, and incubated with 200 nm rhodamine-phalloidin (Molecular Probes; 3000 units/1.5 ml MeOH) in PBS for 1 h at room temperature in the dark to stain filamentous actin. The actin cytoskeleton was visualized using conventional laser excitation and filter sets on a confocal laser scanning microscope (Nikon A1R). Rhodamine staining was visualized using conventional laser excitation and filter sets.

2.8. Mammalian actin staining

The EGFP or EGFP-LI1035 fusion proteins were transiently expressed in HEK293 T cells as described in section 2.6. To assess actin localization, HEK293 T cells were fixed with 3.7% formaldehyde for 30 min at room temperature, followed by extensive washing with PBS and staining with rhodamine-phalloidin and DAPI for 20 min. Confocal microscopy was performed by using a confocal laser scanning microscope (Nikon A1R). GFP, DAPI and rhodamine staining were visualized using conventional laser excitation and filter sets.

3. Results

3.1. Construction of a yeast-basedL. intracellularis protein expression array

To date, the full genome sequences of two pathogenic *L. intracellularis* isolates from pigs (PHE/MN1-00, accession number PRJNA183; N343, accession numberPRJNA175550) have been published. The genome consists of a 1.46 Mb chromosome and three plasmids of 0.03, 0.04, and 0.19 Mb with no prominent differences at the genomic level (Sait et al., 2013).The lack of prominent differences at the genomic level between PHE/MN1-00 and N343 suggested that we could design primers based on the sequence of the reference strain PHE/MN1-00 and amplify the genes from *L. intracellularis*-positive porcine ileal mucosal DNA. Indeed, the gene sequences in our study were the same as those of PHE/MN1-00.

A total of 1419 and 1421 proteins are annotated in PHE/MN1-00 and N343, respectively, more than 25.7% of which are annotated as hypothetical ORFs with an unknown function. Given that the amino acid sequences of most effectors do not display significant similarity to proteins of known function, we targeted all hypothetical genes of unknown function for expression in yeast. The high levels of expression of numerous bacterial genes encoding hypothetical proteins during *L. intracellularis* infection both *in vitro* and *in vivo* suggest that this organism has adopted previously uncharacterized mechanisms of survival and pathogenesis (Vannucci et al., 2012, 2013), which warrants this experiment. Thus, all hypothetical gene fragments (365 genes except for LIC091, encoding 8746 aa) were PCR amplified from *L. intracellularis*-positive porcine ileal mucosal DNA and cloned into the yeast low copy number expression vector pRS416-GAL1. Following plasmid transformation into the yeast strain W303-1A, hypothetical genes were expressed under the control of the galactose-inducible promoter GAL1, and protein expression was confirmed by Western blotting.

3.2. Expression of theL. intracellularis hypothetical gene LI1035 inhibits yeast growth

To identify putative effectors that perturb cellular functions and inhibit yeast growth, yeast cultures were serially diluted and plated onto repressing (2% glucose) and inducing (2% galactose) media. On repressing medium, most yeast strains exhibited similar growth to a control strain containing an empty vector. However, LI1035 was the first identified gene that caused severe growth inhibition when expressed in yeast, inhibiting growth to a degree comparable to that of the positive control, RipI, which is a phytopathogenic virulent effector protein that causes severe growth defects in yeast, as previously described **(**Fig. 1**A)** (Deng et al., 2016).

Effector-driven growth inhibition has been shown to bedependent on the yeast genetic background (Salomon et al., 2011). To ascertain

the function of LI1035 in another genetic background, we transformed the pRS416-GAL1-LI1035 recombinant construct into the *S. cerevisiae* strain BY4741. The same severity of LI1035-induced lethality was observed in the BY4741 genetic background as seen in the W303-1A strain (Fig. 1B). Immunoblot analysis indicated that Rip I and LI1035 were expressed robustly in both W303-1A and BY4741 (Fig. 1C). Taken together, these results indicate that LI1035 targets cellular processes required for yeast growth when expressed under normal conditions from a low copy number expression vector.

3.3. Temperature and sorbitol alter the LI1035-induced growth defects in yeast

The growth temperature and the presence of sorbitol, considered "stressors", have been shown to aid in the identification of effector-mediated growth-retardation traits (Seward et al., 2015). To identify the effect of LI1035 expression on yeast growth at various temperatures on solid media, yeast cultures containing LI1035 or empty vector were serially diluted and plated onto repressing or inducing media in the presence or absence of 0.8 M sorbitol at 20 °C, 30 °C or 37 °C. The growth of yeast expressing LI1035 was monitored and scored based on the highest dilution in which colonies were detected. The results are shown in Fig. 2. We again observed a 100- to 1000-fold decrease in growth when the plates were incubated at 30 °C. However, the decrease in growth was enhanced to 10 to 100-fold in cultures grown at 20 °C and 37 °C, respectively. The growth defects induced by LI1035 expression were ameliorated by sorbitol at all growth temperatures.

Sensitivity to heat stress is correlated with impaired MAPK signaling, cytoskeletal function and membrane transport (Sisko et al., 2006). In contrast, cold sensitivity is correlated with defects in membrane transport in the late secretory pathway and in the assembly of multi-protein complexes. The addition of sorbitol to growth media results in a hypotonic environment, which stabilizes the cell wall under conditions of osmotic stress and rescues the defects in the protein kinase C (PKC)-dependent MAPK pathway and in ER/early Golgi functions (Sisko et al., 2006). The increase in LI1035 toxicity by heat and cold stress and the decrease in LI1035 toxicity by sorbitol are consistent with a primary defect in MAPK signaling pathways.

3.4. Suppression of the LI1035-induced growth defect in yeast

Five MAPK cascades mediating the response to different stimuli have been characterized in yeast: (i) the pheromone response pathway; (ii) the filamentous growth pathway; (iii) the HOG pathway; (iv) the nutrient starvation pathway; and (v) the cell wall integrity (CWI) pathway (Chen and Thorner, 2007). Other studies have shown that the expression of effector proteins in yeast strains with deletions of signaling pathway components can restore wild-type growth if the deleted gene is a target of effector protein activity (Kramer et al., 2007). To

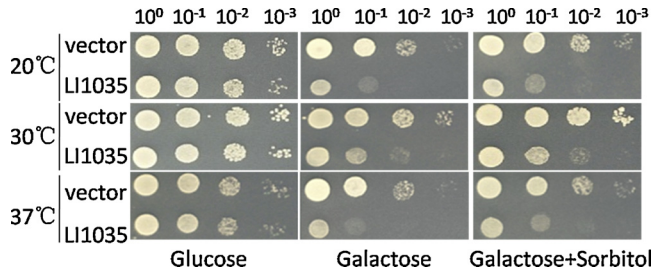

Fig. 2. LI1035-mediated growth inhibition is temperature-sensitive and can be partially relieved by sorbitol.
Ten-fold serial dilutions of cultures were spotted on plates containing either glucose or galactose with or without 0.8 M sorbitol. Plates were incubated for 72 h at the indicated temperatures. Each experiment was repeated three times with similar results.

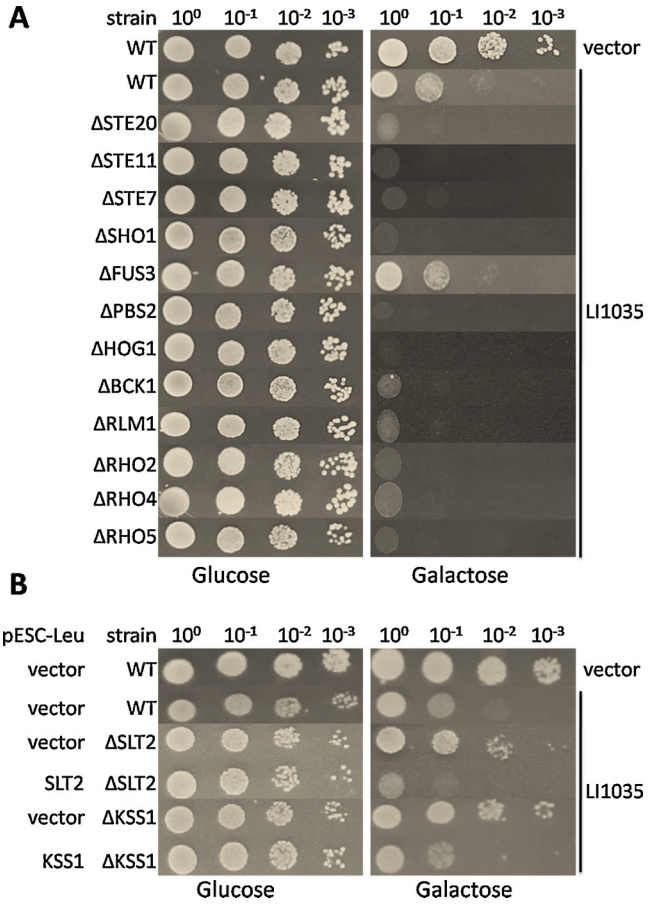

Fig. 3. Genetic approach in yeast to identify functional targets of LI1035.
(A) Deletion of twelve of the 14 MAPK component genes did not suppress LI1035-induced growth inhibition in yeast. Strains with wild-type MAPK component genes or deletion of one nonessential MAPK component gene carrying pRS416-GAL1 or pRS416-GAL1-LI1035 were plated onto SCD-Ura$^-$ or SCG-Ura$^-$, respectively. (B) Deletion of the *kss1 or slt2*gene suppressed the growth inhibition induced by LI1035 in yeast. Wild-type strains and slt2-Δ or kss1-Δ strains carrying pRS416-GAL1 or pRS416-GAL1-LI1035 were plated onto SCD-Ura$^-$ or SCG-Ura$^-$, respectively. Wild-type strains and slt2-Δ or kss1-Δ strains carrying pRS416-GAL1- and pESC-LEU-based plasmids were plated onto SCD-Ura$^-$-Leu$^-$ or SCG-Ura$^-$-Leu$^-$, respectively.

determine whether yeast MAPK pathway components modulate effector toxicity, we transformed LI1035 into yeast strains deleted for components indifferent MAPK signaling pathways and evaluated the growth of these strains under galactose-inducing conditions.

We chose four different MAPK pathways in yeast (pheromone response/mating, filamentous growth, HOG, and CWI) and generated14 strains with deletions in specific, nonessential gene components from the yeast deletion clone library (Thermo Fisher, 95401.H3) (Fig. S1). LI1035 was expressed in each of the yeast mutant strains, which were scored for observable growth defects. No suppression of yeast growth inhibition by deletion of the nonessential genes encoding certain Rho GTPases (*RHO2*, *RHO4*, *RHO5*) and components of the hyperosmotic growth/glycerol (HOG) pathway (*SHO1*, *PBS2*, and *HOG1*) was observed (Fig. 3A). However, the LI1035-mediated yeast growth inhibition phenotype was partially suppressed in the Slt2-Δ and Kss1Δ strains (Fig. 3B).

We next performed complementation analyses to examine whether the restoration of yeast growth in the *slt2*- or kss1- mutant was due to the absence of the *SLT2* or KSS1 gene (Fig. 3B). To this end, the *SLT2* and KSS1 gene coding sequences were cloned into the high copy number plasmid pESC-LEU, and the resulting plasmid was introduced into the yeast strain carrying the pRS416-GAL1-LI1035 plasmid. When

LI1035 was expressed (by galactose induction), yeast growth was again inhibited. Although the growth inhibition observed in the *slt2*- or kss1-mutant was not as severe as that observed in the wild-type strain under LI1035 expression, these results demonstrate that the absence of *SLT2* and KSS1 may be responsible for the suppression phenotype of LI1035.

Kss1 is the MAP Kinase responsible for phosphorylating the transcriptional regulatory protein (Ste12) in the response to pheromones or filamentous growth. Slt2 is the MAP Kinase responsible for phosphorylating the transcriptional regulatory protein (Rlm1 or SBF) in the response to cell wall stress. Notably, neither deletions of genes upstream of KSS1 in the pheromone response pathway and filamentous growth pathway (Ste20, Ste11 and Ste7) nor deletions of genes upstream of SLT2 (*BCK1*) or downstream of SLT2 (Rlm1) in the CWI pathway (Fig. 3A) rescued the growth defect induced by LI1035 expression, suggesting that the specific absence of Kss1 and Slt2 restored the growth ability of yeast.

3.5. LI1035 inhibits theMAPK signaling pathway in yeast

To further investigate the function of LI1035, we exploited the cross-reactivity of mammalian phospho-specificp42/44 MAPK antibodies with phosphorylated SLT2, FUS3 and KSS1 and the cross-reactivity of mammalian phospho-specific p38 MAPK antibodies with phosphorylated HOG1 (Kramer et al., 2007; Siggers and Lesser, 2008).

First, we monitored the effect of LI1035 on CWI pathway activation and SLT2phosphorylation. As shown in Fig. 4A, SLT2 phosphorylation was barely detectable in yeast grown under standard laboratory conditions regardless of LI1035 expression. We then monitored SLT2 phosphorylation in response to heat shock, which was previously shown to activate the CWI pathway (Kramer et al., 2007). As shown in Fig. 4A, LI1035 fully inhibited the phosphorylation of SLT2 and the activation of the CWI pathway.

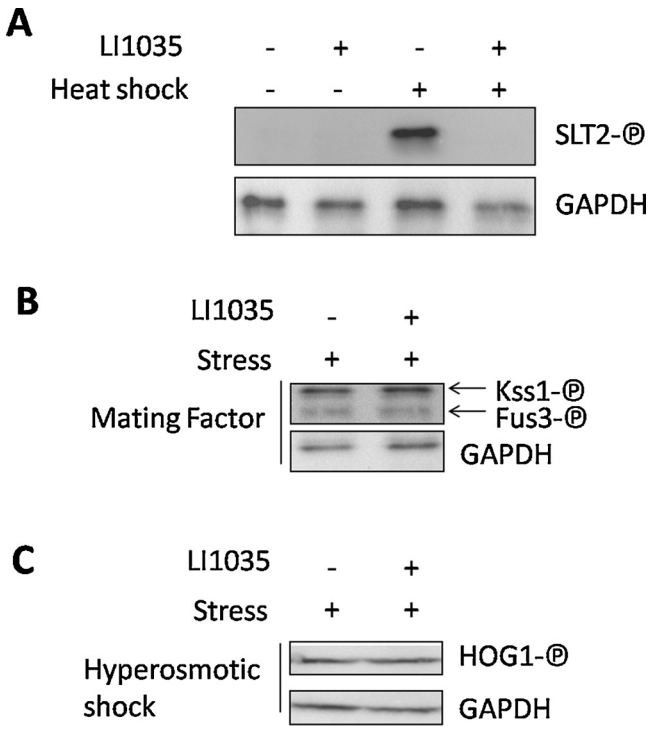

Fig. 4. LI1035 suppresses the CWI pathway in yeast *via* the inhibition of MAPK phosphorylation.
Yeast containing pRS416-GAL1 or pRS416-GAL1-LI1035 were subjected to stress, heat shock (A), a-factor addition (B) and NaCl addition(C) after the induction of LI1035 expression (see the Materials and methods section). Representative immunoblots used to assess the activation of each of the four MAPK signaling pathways—SLT2 (CWI pathway), FUS3 (mating pathway), KSS1 (invasive growth pathway), and HOG1 (HOG pathway)—are shown. The circled P denotes the phosphorylated forms of the proteins. In addition, the blots were probed with the anti-GAPDH antibody as the loading control. Each experiment was conducted at least three times with similar results.

We next monitored the effect of LI1035 on the phosphorylation of FUS1 and KSS1, which is induced by the pheromone MAPK pathway in response to a-factor. As shown in Fig. 4B, the expression of LI1035 did not impair the phosphorylation of FUS1 and KSS1.

Finally, to determine whether LI1035 affects the phosphorylation of HOG1 in yeast in response to NaCl, we tested the effect of LI1035 on the HOG pathway. As shown in Fig. 4C, LI1035 did not impair the phosphorylation of Hog1.

Therefore, LI1035 appears to specifically affect the activity of the CWI pathway in yeast, an effect that likely explains the hypersensitivity of SLT2 deletion strains to LI1035. Because MAPK cascades are highly conserved among eukaryotes and LI1035 is a protein from a swine pathogen, LI1035 likely also targets mammalian MAPK pathways.

3.6. LI1035 attenuates ERK phosphorylation

The observations in yeast suggest that the presence of LI1035 is sufficient to inhibit MAPK phosphorylation by heat shock. Similar experiments were conducted to determine whether this effect occurs also in mammalian cells. Due to the low efficiency of transfection in the IPEC-J2 cell line (data not shown), the HEK293 T cell line was used in this experiment. As shown in Fig. 5A, the phosphorylation of ERK1 and 2 was identical regardless of LI1035 expression. However, LI1035 attenuated ERK phosphorylation in response to EGF stimulation, which induces MAPK signaling (Fig. 5B). Thus, these results indicate that LI1035 specifically inhibits the phosphorylation of MAPK in yeast and mammalian cells.

3.7. LI1035 inhibits actin polarization

The yeast actin cytoskeleton comprises actin cables, used for the delivery of secretory cargo along the mother–bud axis, and cortical actin patches, which are the sites of active endocytosis (Levin, 2011). The actin cytoskeleton is an important component in yeast cell wall maintenance, and the CWI kinase cascade is linked to actin polarization (Levin, 2011). The dramatic effect of LI1035 on the inhibition of the CWI MAPK pathway in response to heat shock prompted us to investigate whether LI1035 can also disrupt actin dynamics and affect polarization in yeast. We monitored actin structures by staining formaldehyde-fixed cells with rhodamine-conjugated phalloidin. As expected in wild-type cells, actin was polarized and mostly localized to the budding pole of dividing cells. In contrast, LI1035 expression in cells led to the collapse of actin cables and cortical patches into an actin-rich patch. These results support our conclusion that LI1035 influences CWI signaling activity and actin polarization (Fig. 6).

3.8. LI1035 disrupts the actin cytoskeleton in mammalian cells

The actin cytoskeleton is essential tocell viability, and the disruption of its structure is most likely detrimental (Liu et al., 2017). To identify whether LI1035 targets the actin cytoskeleton in mammalian cells, a LI1035-EGFP fusion plasmid was constructed, and LI1035 was transiently expressed in HEK293 T cells. As shown in Fig. 7, LI1035 accumulated on the plasma membrane of mammalian cells; ectopic expression of LI1035 caused a detectable rearrangement of the actin cytoskeleton, with increased actin accumulation on the plasma membrane compared with that in cells expressing only EGFP. This finding prompted us to further investigate the mechanism of action of LI1035.

4. Discussion

Although the presence of a T3SSwas revealed by analysis of the *L.*

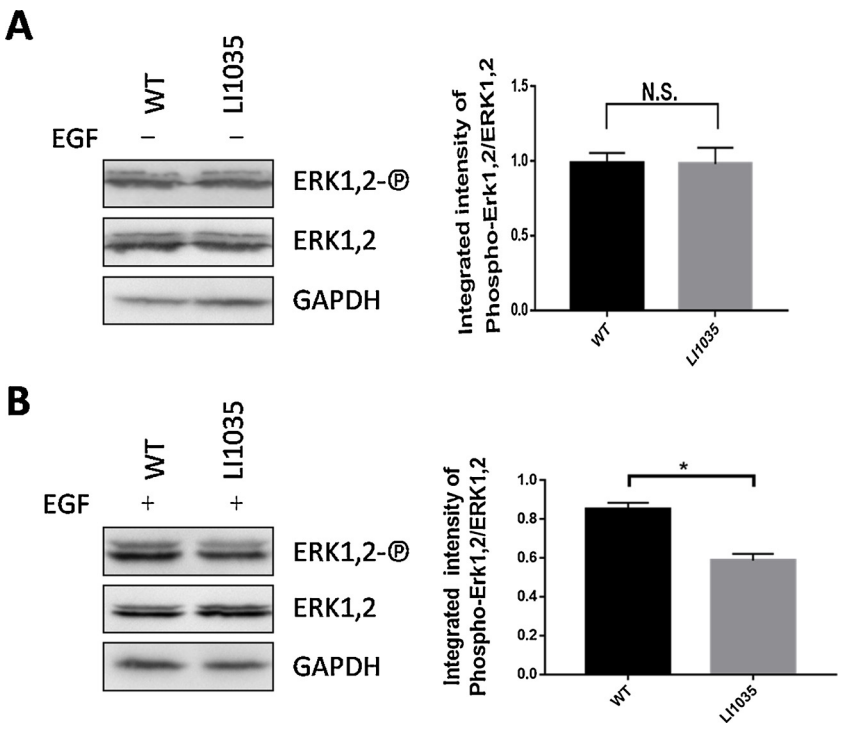

Fig. 5. LI1035 inhibits ERK phosphorylation.
(A)Immunoblots of extracts of HEK293 T cells transfected with pCDNA3.1-LI1035 or pCDNA3.1 for 24 h and then exposed to 50 ngml^{-1} EGF for 30 min to activate ERK. Cell lysates were probed with the designated antibodies. Experiments were conducted at least three times with similar results. (B) The intensity of the bands corresponding to phosphorylated ERK1 and 2 was measured with ImageJ, and the intensity ratio of the bands representing phosphorylated ERK1 and2 and the bands representing total ERK1 and 2 revealed the relative levels of phosphorylated ERK1 and 2in cells of the corresponding samples. All results are from three independent experiments. The error bars represent the standard error of the mean (SEM). *, $p < 0.01$.

intracellularis genome sequence in 2009, the difficulties in axenic (cell-free) medium cultivation and genetic intractability of this organism have hampered the understanding of its pathogenesis and virulence factors (Alberdi et al., 2009). Identification of the effector proteins is a major challenge in understanding T3SS-mediated disease, and knowledge of this arsenal of proteins in bacteria is an essential prerequisite for studying the underlying molecular mechanisms of disease. The high expression level of numerous bacterial genes encoding hypothetical proteins during *L. intracellularis* infection suggests that this organism has acquired survival and pathogenesis mechanisms unique among bacterial pathogens (Vannucci et al., 2012, 2013). We therefore aimed to survey the hypothetical proteins of *L. intracellularis* for potential effector protein activity by using a yeast growth inhibition strategy.

By using a low copy number expression vector, we identified LI1035 as the first *L. intracellularis* protein to be expressed in yeast and to inhibit yeast growth in two genetically distinct yeast strains, W303-1A and BY4741. The LI1035 protein comprises more than 300 amino acids, and no conserved domain has been identified. The increase in LI1035 toxicity by heat and cold stress and the decrease inLI1035 toxicity by sorbitol are consistent with a primary defect in MAPK signaling pathways.

While none of the other 12 of MAPK component deletions rescued the inhibition of yeast growth in the presence of LI1035, deletion of the KSS1 or SLT2 gene suppressed the growth defect induced by LI1035 expression. Complementation of KSS1 or SLT2 deletion reversed this suppression, further supporting the finding that the LI1035-induced growth defect is mediated, either directly or indirectly, through KSS1 and SLT2. However, the growth inhibition was demonstrably weaker in the kss1 and slt2complementation strains than in the strain with chromosomally intact genes, which could be due to the varying expression level of these genes by the plasmid compared with their expression levels in the wild-type strain.

Slt2 is the MAP kinase involved in cell wall biogenesis and maintenance and is regulated by the protein kinase C-mediated CWI MAPK pathway. The suppression of LI1035 toxicity by deletion ofSLT2 but not the downstream RLM1may occur because the negative effect of LI1035 may require its interaction with a gene product(s) whose expression depends on activation *via* Swi4-mediated (but RLM1-independent) mode of gene regulation. LI1035 expression impaired the phosphorylation of SLT2, supporting the hypothesis that LI1035 targets SLT2 in the CWI pathway.

However, the inhibition of growth in wild-type yeast due to LI1035 expression cannot be explained by the inhibition of only the CWI pathway, since deletion strains that no longer express essential components of the CWI pathway do not exhibit growth inhibition under the same conditions. The LI1035 hypersensitivity of the *kss1*deletion strain indicates general inhibition of the yeast MAPK signaling pathways. KSS1 is the MAP kinase that is regulated by the pheromone response pathway and filamentous growth pathway. The finding that the deletion of KSS1 suppressed the toxicity of LI1035 suggests that LI1035 activity may be involved in the pheromone response and filamentous pathways. However, LI1035 did not impair the phosphorylation of three additional yeast MAPKs: Hog1, Kss1, and Fus3.The weak effect on these pathways may be indirect and stem from cross-talk between MAPK pathways, further experiments are required for confirmation.

Many components of MAPK pathways and the mechanisms by which they operate were first identified and characterized in yeast and are now known to have been conserved during the evolution of the entire eukaryotic kingdom(Shan et al., 2007).For example, Kss1 and Slt2 are orthologs of mammalian ERK2 and ERK5, respectively(Shan et al., 2007). Both ERK2 and ERK5 are MAP kinases that are activated in response to growth factors (Morrison, 2012). LI1035 inhibited the phosphorylation of Slt2 in yeast and of ERK1 and 2 in mammalian cells, indicating that the activity of LI1035 is conserved in mammals and yeast.

Inhibition of MAPK phosphorylation is the preferred target of the interaction of T3Es with the eukaryotic cell signaling machinery (Seward et al., 2015). Effectors from both animal and plant pathogens have been shown to inhibit specific steps in the four most well-characterized MAPK signaling pathways in yeast, which contain conserved components and cell surface receptors shared among all eukaryotes (Chen and Thorner, 2007; Seward et al., 2015).The CWI MAPK pathway is the principal signaling pathway maintaining the architecture of the yeast cell wall. The list of diverse effector proteins (*e.g.*, OspF of *Shigella*; VopX of *Vibrio cholerae*; the Icm/Dot effectors of *Coxiella burnetti*; and YopE of *Y*ersinia *pestis*) perturbing the function of the CWI MAPK

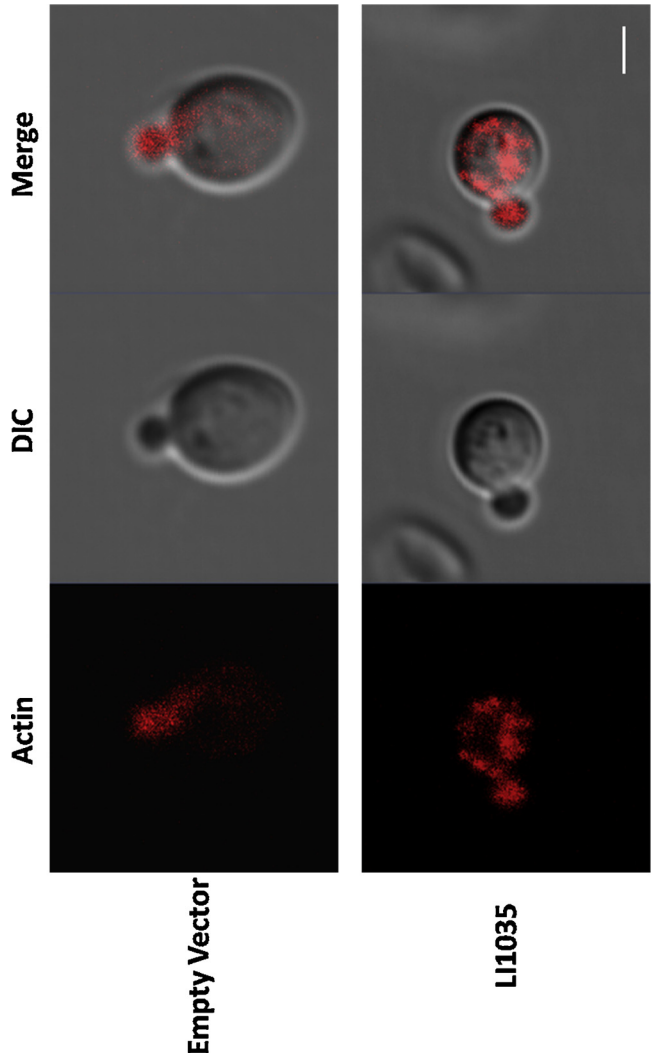

Fig. 6. LI1035 inhibits actin polarization in yeast.
Strains containing pRS416-GAL1 or pRS416-GAL1-LI1035 were induced in selective medium (SCG-Ura$^-$) at 30 °C for 5 h. The actin structure was then visualized using rhodamine-phalloidin and a confocal laser scanning microscope (Nikon A1R). Bars, 2 μm.

pathway in the yeast model system is growing (Kramer et al., 2007; Seward et al., 2015; Ziv et al., 2014). OspF also inhibits the phosphorylation of Slt2 and attenuates the host innate response to *Shigella* infection, indicating that LI1035 may play the same role in *L. intracellularis* infection (Kramer et al., 2007). Our observation of the LI1035-mediated functional modulation of the CWI MAPK pathway will extend the growing list of bacterial effectors targeting this important cellular pathway in yeast.

Actin is a core component of the actin cytoskeleton. In mammalian cells, actin plays a crucial role in diverse cellular processes, including cell migration, cytokinesis, endocytosis and vesicle trafficking (Lamason and Welch, 2017). Therefore, unsurprisingly, many pathogens target actin and/or proteins involved in the regulation of actin activity for their benefit (Popa et al., 2016). Our study highlighted the importance of modulating cellular processes governed by the actin cytoskeleton in the intracellular life cycle of *L. intracellularis*.

Although the screening of all hypothetical *L. intracellularis* proteins that inhibit yeast growth has not yet been completed, our study identified additional hypothetical proteins of *L. intracellularis*, including proteins that demonstrated weak to moderate inhibition and proteins that required yeast-sensitizing agents to induce the phenotype (data not

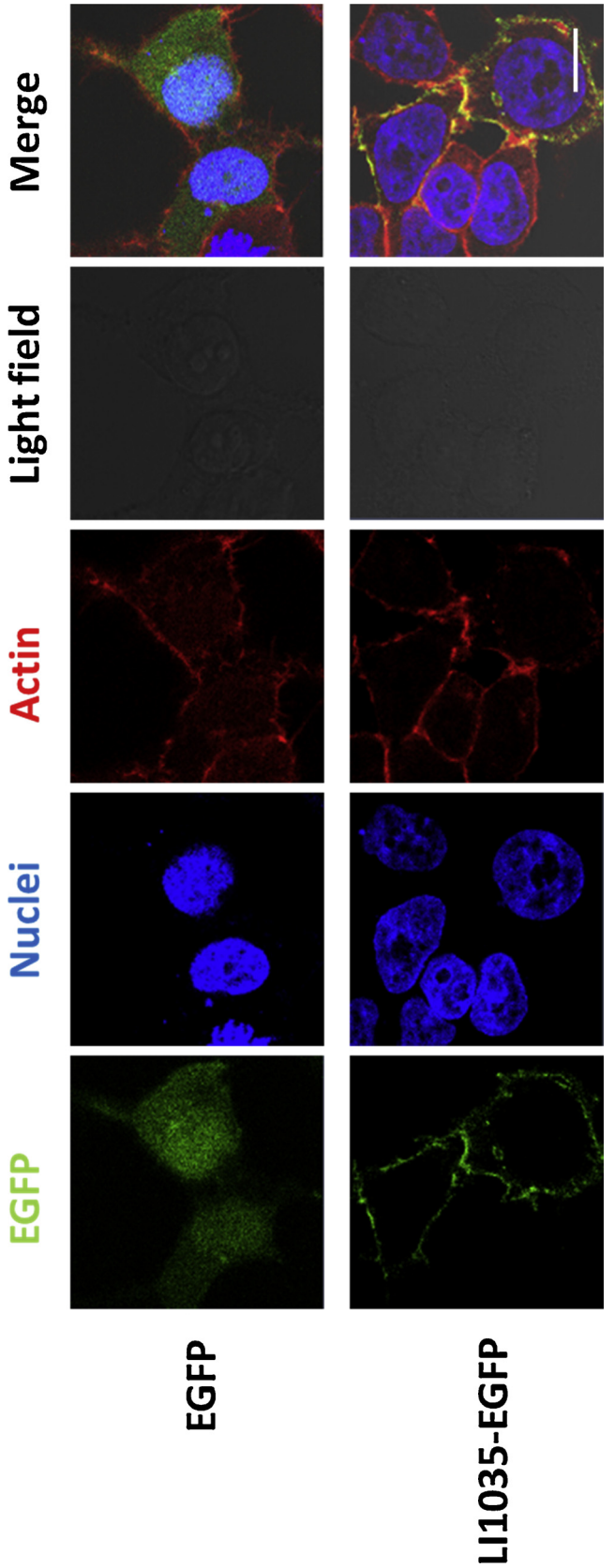

Fig. 7. LI1035 regulates actin organization in mammalian cells.
EGFP and EGFP-tagged LI1035 were transiently expressed in 293 T cells. The cells were fixed in 3.7% paraformaldehyde, nuclei were stained with DAPI and F-actin was stained with phalloidin, and the cells were imaged by a confocal laser scanning microscope (Nikon A1R). Bars, 20 μm.

shown). Collectively, our results indicate that yeast can demonstrate a range of growth defects and be a suitable system for screening *L. intracellularis* effector protein activity. This ability is consistent with the use of yeast to screen effector proteins secreted by other bacterial T3SSs (Popa et al., 2016; Siggers and Lesser, 2008). The yeast phenotypes identified in this study will aid the search for eukaryotic targets of *L. intracellularis* effectors. We expect that the use of yeast in the study of *L. intracellularis* effectors will provide insight into the effector functions and bacterial pathogenesis strategies of organisms that are difficult to genetically manipulate or culture for routine analysis in the laboratory.

The difficulties in axenic (cell-free) media cultivation and genetic intractability of *L. intracellularis* hamper the understanding of the pathogenesis and virulence factors of this pathogen *(*Karuppannan and Opriessnig, 2018*)*. Although yeast growth inhibition is a sensitive and specific reporter of effector protein activity, more studies in *Salmonella*, *Shigella*, or *Yersinia*, which are genetically tractable heterologous host bacteria, are needed to identify bona fide effector proteins translocated into eukaryotic host cells in a T3SS-dependent manner after the identification of these proteins by the yeast screen. We anticipate that advances in genome sequencing and systematic refinements to the growth medium will allow the formulation of an axenic medium that enables host-free cultivation of *L. intracellularis* in the laboratory and the development of sophisticated genetic tools. These innovations will aid the discovery of the underlying principles of bacterial physiology, host–pathogen interactions and mechanisms that govern infection.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.009.

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