Lawsonia intracellularis LI0666 is a new EPIYA effector exported by the Yersinia enterocolitica type III secretion system

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Abstract

Lawsonia intracellularis is the causative agent of proliferative enteropathy. While it harbors genes encoding the entire apparatus required for the type III secretion system (T3SS) and the expression of some of these components has been detected during experimental infection, the identification of L. intracellularis T3SS substrates (effector proteins) has been hampered. The Yersinia T3SS and yeast growth inhibition assays are two important heterologous systems used for the characterization of effector proteins. Bacterial EPIYA effectors are a distinct class of bacterial effectors defined by the presence of EPIYA or the EPIYA-related motif. When delivered into host cells via a T3SS or type IV secretion system, these effectors undergo tyrosine phosphorylation of the EPIYA motif, which enables them to manipulate host cell signaling by promiscuously interacting with multiple SH2 domain-containing proteins. A previous study showed that L. intracellularis LI0666 contains two EPIYA motifs and speculated that this protein could be a T3SS effector. In this study, we show that LI0666 is secreted by Yersinia in a T3SS-dependent manner and inhibits yeast growth. LI0666 is phosphorylated at tyrosine residues in porcine intestinal epithelial cells and in human epithelial cells. Like the archetypal EPIYA effector CagA, the EPIYA-containing region is not required for LI0666 association with yeast and mammalian cell membranes. Our results indicate that LI0666 is an authentic bacterial EPIYA effector. Identification of the tyrosine kinases that are responsible for LI0666 phosphorylation and the SH2 domain-containing host proteins that LI0666 interacts with will help to explore the molecular mechanisms of LI0666 in disease development.

Keywords: Lawsonia intracellularis, EPIYA effectors, Tyrosine phosphorylation, Yersinia T3SS

Introduction

Lawsonia intracellularis is a Gram-negative, obligate, intracellular bacterial pathogen that infects a wide range of animals, mainly pigs and horses, and causes the contagious disease known as proliferative enteropathy [1–3]. Although L. intracellularis was successfully propagated in rat small intestinal cells in 1993 [4], growth of this bacteria in axenic (cell-free) media has not been achieved.

Despite decades of research, the pathogenesis and virulence factors of this organism have not been well-characterized. The sequence of the L. intracellularis genome indicates that it may possess a type III secretion system (T3SS), which could assist the bacterium during cell invasion and evasion of the host’s immune system and induce cellular proliferation [5]. While the expression of some components of this putative T3SS has been detected during experimental infection, the microaerophilic obligate intracellular lifestyle and the genetic dissimilarity between L. intracellularis and other enteric pathogens have hampered the identification of potential T3SS substrates (effector proteins) [5]. Intracellular bacteria typically require the activity of many effector proteins to
remodel the host environment and establish a replicative niche, as exemplified by Chlamydia spp [6], which suggests that effector proteins play a significant role in the pathogenesis of *L. intracellularis*. Given the challenges of culturing *L. intracellularis* under laboratory conditions, currently the most feasible method for effector protein identification is the use of a surrogate system.

The Ysc-Yop T3SS from *Yersinia* was the first T3SS to be characterized, and is considered the archetype [7, 8]. The Yop effector proteins (YopH, YopO/YpkA, YopP/YopJ, YopE, YopM, YopT) and the secretion apparatus used to export them from the bacteria into eukaryotic cells—the injectisome—are all encoded by a 70-kb virulence plasmid called pYV in *Y. enterocolitica* [9]. Secretion and translocation of the Yop effectors are normally triggered by contact with a eukaryotic cell. However, secretion can be artificially induced by chelating Ca²⁺ ions, which leads to a massive release of Yops into the culture supernatant [10]. The coding sequence for a secretion signal is located at the 5' end of each *yop* gene, and the first 15 amino acids of YopE constitute the N-terminal secretion signal [11]. The T3SS needle protein YscF is indispensable for effector protein translocation into host cells [12]. The *Yersinia* T3SS is a genetically tractable heterologous system that has been successfully employed to identify novel T3SS substrates from genetically intractable strains [13].

The budding yeast, *Saccharomyces cerevisiae*, is another important heterologous system used for the screening and functional characterization of effector proteins in a eukaryotic environment, which has been used to study over 100 effectors [14]. Yeast growth inhibition is a sensitive and specific indicator of the activity of effector proteins that perturb conserved cellular processes and has been extensively used as a first step in the search for effector function [3, 14]. Recently, we reported that LI0666, the first putative bacterial EPIYA effector, inhibits yeast growth [15].

Bacterial EPIYA effectors are a distinct class of bacterial effectors defined by the presence of the Glu-Pro-Ile-Tyr-Ala sequence (EPIYA motif) or an EPIYA-related motif, many of which have diverged from the original sequence through multiple duplications [16]. When delivered into host cells via a T3SS or type IV secretion system (T4SS), these effectors undergo tyrosine phosphorylation at the EPIYA motif, which enables them to manipulate host cell signaling by promiscuously interacting with multiple SH2 domain-containing proteins [16]. Since the discovery of the archetypal EPIYA effector, *Helicobacter pylori* CagA, and recognition of the crucial roles that these effectors play in disease manifestations during pathogenic bacterial infection, several additional bacterial EPIYA effectors have been identified. To date, ten EPIYA-motif containing effectors have been identified in six pathogens: *H. pylori* CagA, *Anaplasma phagocytophilum* AnkA, enteropathogenic *Escherichia coli* Tir, *Citrobacter rodentium* Tir, *Chlamydia trachomatis* Tarp, *Haemophilus ducreyi* LspA1 and LspA2, and *Bartonella henselae* BepD, BepE, and BepF [16, 17]. An earlier study showed that CagA translocates into gastric epithelial cells and localizes to the inner surface of the plasma membrane, where it undergoes tyrosine phosphorylation of the EPIYA motif [18]. Further research showed that the EPIYA-containing region of CagA is not required for its membrane association [19, 20].

Recently, using hidden Markov models, Xu et al. showed that EPIYA-containing proteins are significantly overrepresented in intracellular bacteria, extracellular bacteria with T3SS and T4SS, and intracellular protozoan parasites [17]. Analysis of the PHE/MNI-00 sequence using HMM identified 20 *L. intracellularis* proteins that contain the EPIYA motif. Three hypothetical proteins (LI0041, LI0666, and LIC053) contain two copies of the EPIYA motif. The two copies of the EPIYA motif located in LI0666 (EPIYAEIKT Y-149 and EPIYAEIKT Y-186) are similar to the SH2-domain-binding R4 motif in CagA and the Tir motif in Tir (Figure 1A) [17]. LI0666 was predicted to be an extracellular or outer membrane protein in *L. intracellularis* using CELLO v.2.5 [17], and to be a T3SS effector using the EffectiveT3 program [21], which prompted us to explore it in more detail [17].

Here we show that LI0666 is exported by *Yersinia* in a heterologous expression assay, and its expression inhibits yeast growth, both of which indicate that LI0666 is a bacterial EPIYA effector. LI0666 is phosphorylated at the tyrosine residues within its EPIYA motifs in mammalian cells. Although LI0666 localizes to yeast and mammalian cell membranes, similar to CagA, the EPIYA-containing region is not required for membrane association. Identification of the tyrosine kinases that are responsible for LI0666 phosphorylation and the SH2 domain–containing host proteins that LI0666 interacts with will help to explore the molecular mechanisms of LI0666 in disease development.

**Materials and methods**

**Strains and cell lines**

The *E. coli* strain DH5α was used to construct and preserve clonal plasmids and was grown in Luria–Bertani (LB) broth. *Y. enterocolitica* MRS40ΔyopHOPEM (lacking the *Yersinia* Yop T3SS effectors YopH, YopO, YopP, YopE, and YopM, but T3S-proficient) and T3SS-deficient *Y. enterocolitica* MRS40Δyop were routinely grown in brain heart infusion (BHI; TransGen Biotech, Beijing, China) agar, and 200 µg mL⁻¹ ampicillin was added to select expression vectors.
The S. cerevisiae strains W303-1A (MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100) and BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) were grown at 30°C in yeast-peptone-dextrose (YPD) (1% yeast extract, 2% peptone, 2% glucose) broth or agar (2%) or in selective synthetic complete (SC) medium lacking uracil (SC-Ura) to maintain the plasmid and were 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.

The human embryonic kidney (HEK) cell line 293T, human cervical epithelial cell line HeLa, and porcine intestinal epithelial cell line IPEC-J2 (Guangzhou Jenbio Co., Ltd, Guangzhou, China) were cultured in Dulbecco’s modified Eagle medium (DMEM, Solarbio) supplemented with 10% fetal bovine serum (FBS, Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Plasmid construction

The plasmids and primers (synthesized by Sangon Biotech (Shanghai) Co., Ltd., China) used in this study are described in Tables 1 and 2, respectively. The YopE gene (RefSeq: NZ_CP009711.1) from Y. pseudotuberculosis with the coding sequence for a C-terminal HA epitope tag fragment was synthesized and cloned into the EcoRI/SalI sites of the pBAD24 vector under the control of an araBAD promoter. An RplJ gene fragment (RefSeq: NZ_CQAE01000015.1) amplified from genomic DNA of Y. pseudotuberculosis MRS40 or an LI0666 gene fragment (RefSeq: NC_020127.1) amplified from genomic DNA extracted from L. intracellularis—positive porcine ileal mucosa was cloned into the EcoRI/SalI sites of the pBAD24-YopE-HA vector. The coding sequence for the first 40 amino acids of the LI0666 gene was amplified from pBAD24-LI0666-HA and inserted into pBAD24-YopE-HA in-frame with the coding sequence for the YopE C-terminus (YopEΔ15) by homologous recombination. Plasmids were introduced into Yersinia by electroporation.

The yeast expression vector pRS416-GAL1 has been described previously [15]. Fragments of the LI0666 ORF were PCR-amplified and cloned into pRS416-GAL1 to generate recombinant plasmid pRS416-GAL1-LI0666. Fragments of the LI0666 ORF were amplified by PCR with primer pair LI0666-F/LI0666R, which were then sewn together by fusion PCR using primers LI0666-F and LI0666-R. The PCR product was cloned into pRS416-GAL1 to
## Table 1  Plasmids used in this study

| Plasmids | Genotype/Description | Source |
|----------|----------------------|--------|
| pRS416-GAL1 | GAL1 promoter, 3×Flag tag, URA3, ampicillin | [15] |
| pRS416-GAL1-Li0666 | GAL1 promoter, 3×Flag-Li0666, URA3, ampicillin | This study |
| pRS416-GAL1-Li0666Y149/186A | GAL1 promoter, 3×Flag-Li0666Y149/186A, URA3, ampicillin | This study |
| pYES2URA-Rpl | 2 µ, GAL1/GAL10, Rpl, URA3 | [23] |
| pBAD24-YopE-HA | araC promoter, YopE-HA, ampicillin | This study |
| pBAD24-RplJ-HA | araC promoter, RplJ-HA, ampicillin | This study |
| pBAD24-Li0666-HA | araC promoter, Li0666-HA, ampicillin | This study |
| pBAD24-Li0666_N40-YopEΔN15-HA | araC promoter, Li0666_N40-YopEΔN15-HA, ampicillin | This study |
| pYES2URA-EGFP | EGFP gene fragment was inserted into pYES2URA under GAL1 promoter | This study |
| pBAD24-Li0666-YopE_HA | araC promoter, Li0666-YopE_HA, ampicillin | This study |
| pBAD24-Li0666-YopE_HA | araC promoter, Li0666_YopE_HA, ampicillin | This study |
| pBAD24-Li0666-EGFP | Li0666 gene fragment was inserted into pBAD24 under araC promoter | This study |
| pBAD24-Li0666-EGFP | Li0666 gene fragment was inserted into pBAD24 under araC promoter | This study |
| pBAD24-Li0666-EGFP | Li0666 harboring A in place of Y at position 149 | This study |
| pBAD24-Li0666-EGFP | Li0666 harboring A in place of Y at position 186 | This study |
| pBAD24-Li0666-EGFP | Li0666 harboring deletion for 1–39 | This study |
| pBAD24-Li0666-EGFP | Li0666 harboring deletion for 141–204 | This study |
| pEGFP-C1 | CMV, Neomycin, kanamycin, EGFP | Clontech |
| pEGFP-C1-Li0666 | Li0666 gene fragment was inserted into pEGFP-C1 in frame with EGFP at the N-terminal | This study |
| pEGFP-C1-Li0666 | Li0666 gene fragment was inserted into pEGFP-C1 in frame with EGFP at the N-terminal | This study |
| pEGFP-C1-Li0666 | Li0666 harboring deletion for 141–204 was inserted into pEGFP-C1 in frame with EGFP at the N-terminal | This study |

## Table 2  Primers used in this study

| Primer | Sequence (restriction enzyme sites are underlined) | Restriction enzyme |
|--------|---------------------------------------------------|--------------------|
| Li0666-F | ATTACAAGGATGACGATGACATGGGCAGGGGCGCGATGAAATTTCAATTTAAA | NotI |
| Li0666-R | GCGTGAATGATGGCGACATGACTATACATGACGCGAGTTTGTATTTCCCTTTT | XhoI |
| Li0666Y149A-F | AACCCCTATCATGCCCATTTTGTGGCAAACTGTGTTATTTAC | Fusion PCR |
| Li0666Y149A-R | TGCAAGAATAGGCCGTAGAAGGGTT | |
| Li0666Y186A-F | CCACTGATTACAGACACGCCATGGGCTAGCGGATCGACATTC | |
| Li0666Y186A-R | AGCAAGATCGGTTCGTACATGTTACATGTGG | |
| pBAD24-YopE-F | CCCGAATCTGAAATATATCATT | EcoRI |
| pBAD24-YopE-HA-R | CCCGAATCTGAAATATATCATT | HindIII |
| pBAD24-RplJ-F | GGTTTTTGGCTGAGCAGGAGGAATTCCGAGCTAAATCTTCA | SalI |
| pBAD24-RplJ-HA-R | ATCTGGTACGTGATGGTATGTGACAGCCTCCTTTCTGATC | SalI |
| pBAD24-Li0666-F | GTTTTTTGGCTGAGCAGGAGGAATTCCGAGCTAAATCTTCA | SalI |
| pBAD24-Li0666-HA-R | ATCTGGTACGTGATGGTATGTGACAGCCTCCTTTCTGATC | SalI |
| pBAD24-Li0666_N40-R | GCTAGATCTGACAGACAGCCTCCTTTTGAAT | BamHI |
| EGFP-F | ccgaATTCGACGGTACGTCGAGGAGGAATTCCGAGCTAAATCTTCA | EcoRI |
| EGFP-R | cccGAGCTCGAGCTTACTCTGATAGCCTGTCG | XhoI |
| Li0666-ΔN39-EGFP-F | CGGACGATCCGAGGAGGAATTCCGAGCTAAATCTTCA | BamHI |
| Li0666-ΔC64-EGFP-R | AGCAACCGGAGGAGGAATTCCGAGCTAAATCTTCA | BamHI |
| Li0666-EGFP-F | AAGGTACCTAGGATCCGAGGAGGAATTCC | BamHI |
| Li0666-EGFP-R | AGCAACCGGAGGAGGAATTCCGAGCTAAATCTTCA | BamHI |
| pEGFP-C1-Li0666-F | ATGGATGACGATGACATGGGCAGGGGCGCGATGAAATTTCAATTTAAA | EcoRI |
| pEGFP-C1-Li0666-R | ATGGATGACGATGACATGGGCAGGGGCGCGATGAAATTTCAATTTAAA | EcoRI |
| pEGFP-C1-Li0666-ΔC64-R | CGGCGATCCGAGGAGGAATTCCGAGCTAAATCTTCA | BamHI |
create the pRS416-GAL1-LI0666_149/186A plasmid. A full-length EGFP gene was amplified from the pEGFP-C1 plasmid and the amplified DNA products were cloned into pYES2/NTA to construct the pYES2/NTA-EGFP plasmid. DNA fragments encoding full-length LI0666, LI0666_149/186A, or truncated LI0666 genes were cloned into the pYES2/NTA-EGFP and pEGFP-C1 plasmids individually.

**Yersinia T3S assays**

T3SS-competent *Y. enterocolitica* MRS40ΔyopHOPEM and T3SS-null *Y. enterocolitica* MRS40ΔyscF were used in the T3S assays, which were performed as previously described [22]. *Yersinia* was cultivated in BHI supplemented with either 5 mM EGTA and 20 mM MgCl₂ (−Ca²⁺) or 5 mM CaCl₂ (+Ca²⁺). Gene expression was induced by adding 0.2% L-arabinose to the culture, and proteins in bacterial pellets and culture supernatants were analyzed by immunoblotting with an anti-HA antibody.

**Yeast growth assays**

pRS416-GAL1 vectors carrying LI0666 were introduced to yeast strains W303-1A and BY4741. Yeast growth assays were performed as described previously [15]. Briefly, the yeast strains were grown overnight in liquid selective medium containing glucose, then washed and diluted to an OD₆₀₀ of 1. Each strain was then ten-fold serially diluted four times and spotted (5 μL) onto SCD-Ura plates and the plates were incubated at 30 °C for 2–4 days.

**Yeast phosphorylation assays**

Yeast strains expressing wild-type or mutated LI0666 were grown in selective SCD-Ura medium at 30 °C until mid-log phase. The cells were then pelleted, washed, and resuspended in selective induction medium (SCG-Ura). After 12 h of induction, the cells were pelleted, washed, and cultured in selective induction medium (2% galactose, 1% raffinose). After 12 h of induction, cells were fixed in 3.7% formaldehyde for 30 min at room temperature and washed twice with PBS containing 1 mg mL⁻¹ BSA, and GFP was visualized using conventional laser scanning microscope (Nikon A1R).

**Fluorescence microscopy to determine subcellular localization in yeast**

Plasmids were transformed into yeast strain W303-1A, which was then grown in SCD-Ura medium at 30°C to exponential phase (OD₆₀₀ = 0.8–1.0). Cells were pelleted, washed, and cultured in selective induction medium (2% galactose, 1% raffinose). After 12 h of induction, cells were fixed in 3.7% formaldehyde for 30 min at room temperature and washed twice with PBS containing 1 mg mL⁻¹ BSA, and GFP was visualized using conventional laser excitation and filter sets on a confocal laser scanning microscope (Nikon A1R).

**Fluorescence microscopy to determine subcellular localization in mammalian cells**

HEK293T cells were seeded at 2.5 × 10⁵ cells/well in a 6-well tissue culture plate. Plasmids were transfected into the HEK293T cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol, and the plates were incubated at 37 °C for 24 h. Next, the transfected cells were grown on coverslips, fixed in 4% paraformaldehyde (PFA, Sigma) for 10 min, and then permeabilized in 0.2% Triton X-100 for 10 min. All steps were performed in 1 × PBS (phosphate buffered saline, Fisher). Coverslips were mounted with DAPI (Sangon Biotech, Shanghai Co., Ltd.), and fluorescence was observed using a fluorescence microscope.

**Results**

Identification of LI0666 as a type III secretion system substrate using the heterologous *Y. enterocolitica* system

LI0666 was predicted to be a T3SS effector by the EffectiveT3 program, which prompted us to investigate it in further detail. Due to the difficulties associated with *L. intracellularis* in vitro cultivation and the lack of a genetic system for this organism, we investigated LI0666 secretion in an alternative host. Hence, we expressed LI0666 in T3S–competent (WT) or –null (∆yscF) *Y. enterocolitica* as an HA-tagged protein under the control of an arabinose-inducible promoter. RplJ (an endogenous ribosomal protein) and YopE (an endogenous T3SS substrate) were used as the negative and positive controls,
Ectopic expression of LI0666 inhibits yeast growth, and this effect is dependent on the tyrosine residues in the EPIYA motifs

To determine whether LI0666 perturbs cellular functions and inhibits yeast growth, Flag-tagged LI0666 was expressed from an inducible promoter in S. cerevisiae strain W303-1A. Under inducing conditions, LI0666 caused severe growth inhibition, comparable to that of the positive control, Ripl, a virulent phytopathogenic effector protein, which was expressed from the high-copy number vector pESC-URA (Figure 2A) [23]. To ascertain the function of LI0666 in another genetic background, we transformed the pRS416-Gal1-3×flag-LI0666 plasmid into strain BY4741. Comparable lethality was observed in BY4741 and W303-1A (Figure 2A). The activity of bacterial EPIYA effectors largely depends on tyrosine phosphorylation. To ascertain the contribution of tyrosine to the LI0666-mediated toxicity observed in the yeast system, we replaced the tyrosine residue within the LI0666 EPIYA motifs with an alanine residue and evaluated the lethality of LI0666-mediated toxicity observed in the yeast system (Figure 2A).

Although S. cerevisiae lacks endogenous tyrosine kinases, it does express some dual-specificity Ser/Thr kinases that are also committed to tyrosine phosphorylation [24]. To investigate the level of LI0666 expression and tyrosine phosphorylation, yeast cell lysates were prepared and immunoblotted with an anti-FLAG and an anti-phosphotyrosine antibody. Our results show that, when expressed in yeast, WT LI0666 was stable and formed a tetramer (the molecular weight of the monomer is 24 kDa) or a large complex, while LI0666Y149/184A did not polymerize and was unstable. Moreover, no tyrosine phosphorylation of LI0666 was detected (Figure 2B). These results suggest that the cytotoxicity of LI0666 in yeast is dependent on the tyrosine residue in the EPIYA motif, but not on tyrosine phosphorylation.

LI0666 EPIYA motifs undergo tyrosine phosphorylation in mammalian cells

To investigate whether LI0666 is tyrosine-phosphorylated at its EPIYA motifs in mammalian cells, constructs encoding EGFP-LI0666 and EGFP-LI0666Y149/184A were transiently transfected into HEK293T cells, which were then immunoblotted with an anti-GFP antibody and a 4G10 antibody. As shown in Figure 3A, EGFP-LI0666 was expressed and efficiently tyrosine-phosphorylated in HEK293T cells, whereas EGFP-LI0666Y149/184A was expressed but not phosphorylated. This phenotype was also observed in HeLa cells and in IPEC-J2 cells (the natural host for L. intracellularis) (Figures 3B and C). These results indicate that LI0666 undergoes tyrosine phosphorylation in porcine epithelial cell cytoplasm when delivered by a T3SS, and that this phosphorylation can also be carried out by kinases in human epithelial cells.

EPIYA motifs are not required for membrane localization of LI0666 in S. cerevisiae or in mammalian cells

We next sought to determine the subcellular localization of LI0666 in yeast. To do this, we constructed a plasmid encoding EGFP fused to the C-terminus of LI0666 and transformed it into yeast. The tagged LI0666 protein localized to the plasma membrane (Figure 4A). The LI0666Y149/184A mutant also localized to the plasma membrane, indicating that membrane localization of LI0666 is independent of the tyrosine residue within the EPIYA motifs. To identify the region of LI0666 that is responsible for membrane localization, we generated constructs expressing LI0666 mutants that lack the N-terminal region (LI0666ΔN39) or C-terminal region (harboring two EPIYA motifs) (LI0666ΔC64). LI0666ΔN39 and LI0666ΔC64 respectively. Equal amounts of cultured cells expressing LI0666-HA, YopE-HA, or RplJ-HA were centrifuged and fractionated into cell-free culture supernatants and Yersinia-containing whole-cell pellets. Immunoblot analysis of WT cultures revealed essentially equal LI0666-specific signals in +Ca2+ (repressive) and −Ca2+ (inductive) cell pellet fractions, but LI0666 was only detected in the supernatant in T3SS-inductive but not-repressive conditions, as was the positive control YopE. This outcome was not due to bacterial lysis, because the Yersinia cytoplasmic protein RplJ was detected only in whole-cell pellet samples and not in the supernatant fractions (Figure 1B). LI0666 was not detected in the supernatant from the ∆yscF strain, which also confirmed that secretion of LI0666 was dependent on a functional T3SS in Yersinia (Figure 1B).

To further identify the T3S signal of the LI0666 protein, we analyzed secretion of the first 40 amino acids of LI0666 fused to the YopEΔAN15. LI0666N40-YopEΔAN15 was detected in supernatant from WT cultures grown under T3SS-inductive but not-repressive conditions (Figure 1B). LI0666N40-YopEΔAN15 was not detected in the supernatant from the ∆yscF strain, nor was the positive control YopE, which also confirmed that secretion of LI0666 was dependent on a functional T3SS in Yersinia (Figure 1B).
A

| Strains  | Vector    | Expressed protein | 10^0    | 10^-1 | 10^-2 | 10^-3 | 10^0    | 10^-1 | 10^-2 | 10^-3 |
|----------|-----------|-------------------|---------|-------|-------|--------|---------|-------|-------|-------|
| W303-1A  | pRS416-Gal1 | none              |         |       |       |        |         |       |       |       |
| W303-1A  | pYES2     | RipI              |         |       |       |        |         |       |       |       |
| W303-1A  | pRS416-Gal1 | 3×Flag-LI0666^WT  |         |       |       |        |         |       |       |       |
| W303-1A  | pRS416-Gal1 | 3×Flag-LI0666^Y149/186A |       |       |       |        |         |       |       |       |
| BY4741   | pRS416-Gal1 | 3×Flag-LI0666^WT  |         |       |       |        |         |       |       |       |

Glucose  Galactose

B

| BY4741 | W303-1A |
|--------|---------|
| Y149/186A | Y149/186A |
| LI0666 WT | LI0666 WT |
|          |          |
| 100  | 10^-1 | 10^-2 | 10^-3 |
| 50   | 10^-1 | 10^-2 | 10^-3 |
| 40   | 10^-1 | 10^-2 | 10^-3 |
| 35   | 10^-1 | 10^-2 | 10^-3 |
| 170  | 130   | 100   |
| 70   | 55    | 40    |
| 35   | 25    |       |

IB: anti-Flag  IB: 4G10

Figure 2  LI0666 expression modulates yeast growth. A LI0666 expression inhibits yeast growth. W303-1A and BY4741 yeast strains carrying the yeast expression vector pRS416-GAL1, either empty or encoding LI0666 or LI0666^Y149/186A, with a N-terminal 3× Flag, were grown overnight in repressing medium (2% glucose). Cultures were then normalized to OD600 1.0, and serial tenfold dilutions were grown at 30 °C for 2 and 3 days in repressing and inducing medium (2% galactose and 1% raffinose), respectively. W303-1A yeast carrying the yeast expression vector pYES2/NT-Ripl was used as the positive control. B Induction of expression was verified by Western blotting using an anti-Flag antibody and an anti-phosphotyrosine antibody (4G10) for 3× Flag-LI0666 and 3× Flag-LI0666^Y149/186A.
both localized to the plasma membrane, indicating that, like CagA, stable association of LI0666 with the membrane does not require the EPIYA-containing region (Figure 4A).

To further investigate the subcellular localization of LI0666 in mammalian cells, vectors expressing LI0666 fused to EGFP were transiently transfected into HEK293T cells. EGFP–LI0666 was localized to the plasma membrane, as well as to an intracellular component that appeared to be vesicular. This distribution pattern was similar to that observed for both LI0666Y149/184A and EGFP-LI0666ΔC64, indicating that neither the tyrosine residue nor the EPIYA-containing region influences the intracellular distribution of LI0666 (Figure 4B). These results indicate that, like CagA, the membrane localization signal of LI0666 is located within the middle portion of the protein and is independent of the EPIYA-containing region.

Discussion
In the present work, we demonstrate that L. intracellularis LI0666, a protein that contains two EPIYA motifs, appears to be a T3SS effector, because it is secreted by the Y. enterocolitica T3SS secretion system and inhibits yeast growth. LI0666 localized to the plasma membrane of yeast and mammalian cells, and neither EPIYA tyrosine phosphorylation nor the EPIYA-containing region were required for this localization pattern. Furthermore, LI0666 underwent tyrosine phosphorylation of the EPIYA motif in mammalian cells.

We previously reported that LI1035 inhibits yeast growth and is the first putative L. intracellularis effector [15]. In the current study, we found that LI0666 was exported by the Yersinia T3SS and also inhibited yeast growth, indicating that it is most likely an authentic effector protein. LI0666-mediated inhibition of yeast growth was not dependent on tyrosine phosphorylation, but did require the presence of the tyrosine residues in the EPIYA motifs, which may be related to their effect on protein polymerization or stability. We also expressed two other EPIYA-containing proteins, LI0041 and LIC053, in S. cerevisiae, and found that their expression did not alter the yeast growth (data not shown). Further experiments are needed to test these two proteins.

Previous reports indicated that the Domain II in the middle portion of CagA, but not the EPIYA containing region, is required for its membrane association [19, 20]. LI0666 was found to localized to the plasma membrane in yeast and mammalian cells in our experiment, which also independent of the EPIYA-containing region. Therefore, elucidating the function of LI0666 may provide further insights into the mechanisms underlying the generation and evolution of bacterial EPIYA effectors during the coevolution of bacterial pathogens with their hosts.

Here we identified LI0666 as a new L. intracellularis EPIYA effector. Identification of the tyrosine kinase members responsible for LI0666 phosphorylation and the SH2 domain-containing host proteins that LI0666 interacts with will help to explore the molecular mechanisms of LI0666 in disease development.
Acknowledgements
We are very grateful to Prof. Dr Feng Shao, National Institute of Biological Sciences, Beijing, China for providing the Y. enterocolitica MRS40ΔyopHOPEM and Y. enterocolitica MRS40ΔyspF strain and pBAD24 plasmid.

Author contributions
CC, YY and ZZ performed the research and data analysis. YD, QZ, XA and FL designed the experiments. FL wrote the paper. All authors read and approved the final manuscript.

Funding
This work was supported by the grants from the National Natural Science Foundation of China (Grant No. 31802213 and 31660720) and from the education department of Jiangxi province (Grant No. GJJ190175).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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Received: 3 January 2022 Accepted: 4 March 2022

Published online: 04 June 2022

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Figure 4 Subcellular localization of GFP-LI0666 fusion proteins in S. cerevisiae and mammalian cells. A Cells were cultured for 10 h under inducing conditions in galactose medium at 30 °C. Empty vector (pYES2/NTA encoding EGFP) was used as the negative control. LI0666-EGFP, LI0666Y149/186A-EGFP, LI0666ΔN39-EGFP and LI0666ΔC64-EGFP, which is a deletion mutant of LI0666 lacking the C-terminal 64 amino acids, was expressed in yeast respectively. B EGFP-LI0666, EGFP-LI0666Y149/186A and EGFP-LI0666ΔC64 were transfected into HEK293T cells. After incubation at 37 °C for 24 h, the nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole), and fluorescence was observed using a fluorescence microscope.
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