Lysobacter enzymogenes Employs Diverse Genes for Inhibiting Hypha Growth and Spore Germination of Soybean Fungal Pathogens

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ABSTRACT

Lysobacter enzymogenes strain C3 (LeC3) is a potential biocontrol agent for plant diseases caused by fungi and oomycetes. Understanding the interaction between LeC3 and soybean pathogens at the molecular level could help improve its biocontrol efficacy. In this study, we obtained mutants with decreased abilities in inhibiting hypha growth of the white mold pathogen *Sclerotinia sclerotiorum*. Insertion sites for 50 mutants, which no longer inhibited *S. sclerotiorum* hypha growth in dual cultural assay, were determined and seven mutants were selected for further characterization. These seven mutants also completely lost their abilities in suppressing spore germination of *Fusarium virguliforme*, the causal agent of soybean sudden death syndrome. Furthermore, mutation of the seven genes, which encode

diguanylate cyclase, transcriptional regulators from the TetR family, hemolysin III family channel protein, type IV secretion system VirB10 protein, phenol hydroxylase, and phosphoadenosine phosphosulfate reductase, respectively, led to reduced production or secretion of four extracellular enzymes and heat-stable antifungal factor (HSAF). These results suggest that these seven genes play important roles in *L. enzymogenes* in suppressing hypha growth and spore germination of fungal pathogens, probably by influencing production or secretion of extracellular enzymes and HSAF.

Keywords: bacteriology, biocontrol, c-di-GMP, genetics and resistance, molecular mechanism, type 4 secretion system

Soybean (*Glycine max*), an important protein and oil provider, is crucial in global food security (Ainsworth et al. 2012). As the world's biggest soybean exporter, the United States produced about 4.6 billion bushels of soybean, valued at \$39 billion, in 2018 (SoyStats 2019, <http://soystats.com/>). Despite the increased acreage and yield, yield losses caused by soybean diseases can be tremendous. It was estimated that plant diseases reduced U.S. soybean yield by about 495 million bushels, approximately 12.5% of the 2014 annual yield and valued at about \$5 billion (<http://extension.cropsciences.illinois.edu/fieldcrops/diseases/>, estimates of soybean yield reductions caused by diseases in the United States). *Sclerotinia* stem rot (SSR) and sudden death syndrome (SDS) are two of the five most damaging soybean diseases, leading to 37- and 68-million-bushel soybean losses in the United States in 2014, respectively. SSR (white mold) is a stem disease caused by *Sclerotinia sclerotiorum*, whereas the causal agent of SDS is the soilborne fungus *Fusarium virguliforme* (*F. solani* f. sp. *glycines*) (Hartman et al. 2015; Smith and Boland 1989). Integrated management for SSR and SDS includes selection of resistant varieties, nonhost crop rotation, modest tillage, canopy management, fungicide, and biological controls, as well as delayed planting date and control of soybean cyst nematode (Mueller et al. 2016). However, crop rotation with corn is not effective for SDS because *F. virguliforme* can be harbored in corn residue, resulting in SDS

outbreaks even after several years of continuous corn planting (Mueller et al. 2016).

Fungicide application is one of the most common disease-controlling methods, but the emergence of pathogen resistance has diminished its efficacy (Brent and Hollomon 1998). To date, fungicide resistance has been reported across a wide range of fungal pathogens, including *Fusarium*, *Sclerotinia*, *Verticillium*, *Penicillium*, *Botrytis*, *Cercospora*, *Alternaria*, *Colletotrichum*, *Sphaerotheca*, *Mycosphaerella*, *Aspergillus*, *Phytophthora*, *Pythium*, and *Ustilago* (Agrios 2005; Jo et al. 2008). On the other hand, most fungicides control plant diseases for their direct toxicity to pathogens by inhibiting cell wall biosynthesis, damaging cell membranes, and inactivating essential enzymes or certain proteins within target pathogens (Andersen et al. 2002; Hartman et al. 2014; Martínez-Toledo et al. 1998; Ochoa-Acuña et al. 2009; Taxvig et al. 2007; Ward et al. 2006). However, increased public concerns about residual fungicides in food and water have led to intensive efforts to develop more sustainable and environment-friendly control methods, such as biological control.

Normally, biological control involves the development of introduced antagonistic microorganisms and uses their natural antagonism to mitigate plant diseases. Previous studies summed up five widely accepted biocontrol mechanisms: mycoparasitism, antibiosis, extracellular enzymes, competition, and induced systematic resistance (Heydari and Pessarakli 2010; Narayanasamy 2013; Whipps 2001). Most commonly used biological control agents (BCAs) include *Gliocladium virens*, *Trichoderma harzianum*, *Agrobacterium radiobacter* K-84, *Pseudomonas fluorescens*, and *Bacillus subtilis* (Agrios 2005). In recent years, some gram-negative bacteria from the genus *Lysobacter* have emerged as novel BCAs against plant diseases (Wang et al. 2013). The *Lysobacter* species are ubiquitous in soil, aquatic environments, and some extreme conditions including hydrothermal vents, volcanic ash, tar pits, and compost sludge (Christensen and Cook 1978; Reichenbach 2006; Sullivan et al. 2003). *Lysobacter* sp. belongs to the family Xanthomonadaceae and was first proposed by Christensen and Cook in 1978 (de Bruijn et al. 2015; Hayward et al. 2010; Reichenbach

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2006). *Lysobacter* was first noted as a source of lytic enzymes with biodegradation abilities in organic matter recycling (Tigerstrom 1980). In the late 1990s, it emerged as novel BCA against many plant diseases (Koenning and Wrather 2010; Wang et al. 2013).

Lysobacter enzymogenes strain C3 (LeC3), which was first isolated from Kentucky bluegrass foliage in Nebraska in 1998, suppresses brown patch (*Rhizoctonia solani* Kuhn) (Giesler and Yuen 1998) and leaf spot (*Bipolaris sorokiniana*) on tall fescue (Zhang and Yuen 1999), *Pythium* damping-off of sugar beet (Palumbo et al. 2005), bean rust (*Uromyces appendiculatus*) (Yuen et al. 2001), *Fusarium* head blight of wheat (*Fusarium graminearum*) (Jochum et al. 2006), sugar beet cyst nematode (*Heterodera schachtii*), root-knot nematode (*Meloidogyne javanica*), and root lesion nematode (*Pratylenchus penetrans*) (Chen et al. 2006). Previous research confirmed the broad-spectrum activities of LeC3 in suppressing hypha growth and spore germination of eight fungal or oomycete pathogens on soybeans, including *S. sclerotiorum*, *R. solani*, *F. virguliforme*, *Cercospora sojina*, *Macrophomina phaseolina*, *Phytophthora sojae*, *Pythium sylvaticum*, and *Septoria glycines* (Nian 2015). In LeC3, genes for antibiotics and extracellular enzymes, such as chitinase, β -1,3-glucanase, heat-stable antifungal factor (HSAF, a broad-spectrum antifungal antibiotic), and WAP-8294A2, have been identified and characterized (Palumbo et al. 2005; Qian et al. 2012; Yu et al. 2006; Zhang et al. 2011). Moreover, regulatory systems in *L. enzymogenes*, including the Clp global regulator and quorum sensing systems mediated by diffusible signal factors or diffusible factors, were found to be involved in the modulation of its biocontrol-related characteristics (Chen et al. 2018; Kobayashi et al. 2005; Qian et al. 2013). The purpose of our study is to further identify and characterize novel genes involved in biocontrol activities of LeC3 in suppressing hypha growth and spore germination of important fungal pathogens of soybean.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli*

strain DH10 β , used for plasmid construction, was routinely grown in Luria-Bertani (LB) medium at 37°C. Tryptic soy medium (10% trypticase soy broth, 1.5 g/liter of tryptone, 0.5 g/liter of soytone, and 0.5 g/liter of NaCl) was used for culturing LeC3 and its derived strains at 28°C (Yu and Zhao 2019). Antibiotics were used as required at the following concentrations unless otherwise noted: gentamicin at 30 μ g/ml and kanamycin (Km) at 100 μ g/ml. Primers used in this study for inverse PCR, mutant confirmation, cloning, and sequencing are listed in Supplementary Table S1.

Dual culture assay. Dual cultural assay was used to determine the inhibition of *S. sclerotiorum* hypha growth by LeC3 and its derivative strains, as described previously (Nian 2015). In brief, 5-mm-diameter discs of fresh-grown *S. sclerotiorum* BF0934, originally obtained from Dr. Carl Bradley's lab at University of Illinois, were placed in the center of 25% potato dextrose agar (PDA) or 10% trypticase soy agar plate. Bacterial suspensions (5 μ l, OD₆₀₀ = 0.2 unless otherwise noted) were pipetted into the same plate about 2.5 cm away from the fungal disc. After incubation at 28°C, fungal hyphae reached plate edges, and the diameters of inhibition zones around LeC3 or its derived mutants were measured. The experiments were repeated at least twice in triplicate.

Construction of *L. enzymogenes* transposon (Tn) mutant library and identification of Tn insertion sites. A transposon mutagenesis kit was used for random mutagenesis according to the manufacturer's instructions. Briefly, to prepare competent cells, an overnight bacterial culture was subcultured to exponential phase in LB amended with Km. Cells were then harvested, washed with cold sterile water, and transformed with 1 μ l of transposome by electroporation. After 3 h of incubation at 28°C, transformants were plated on LB amended with 2,000 μ g/ml Km (Yu and Zhao 2019). A total of 948 colonies was picked and screened via dual culture assay. Mutants with reduced inhibition zones were selected and stored at -80°C.

Inverse PCR or random amplification of transposon ends (RATE) PCR was used to determine Tn5 insertion site. Inverse PCR was performed as previously described (Lee et al. 2018). Briefly,

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Description	Reference or source
Strains		
<i>Escherichia coli</i> DH10B	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80/ <i>lacZ</i> AM15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara leu</i>) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>nupG</i> λ -	Invitrogen (Carlsbad, CA, U.S.A.)
LeC3 ^a	Wild type, isolated from Kentucky bluegrass foliage, Nebraska, Km ^R	(Giesler and Yuen 1998)
<i>dgc</i>	Transposon insertion mutant in gene <i>GLE_0067</i> (encoding one diguanylate cyclase), Km ^R	This study
<i>tetR1</i>	Transposon insertion mutant in gene <i>GLE_0809</i> (encoding one transcriptional regulator from the TetR family), Km ^R	This study
<i>tetR2</i>	Transposon insertion mutant in gene <i>GLE_2933</i> (encoding one transcriptional regulator from the TetR family) and gene <i>GLE_2934</i> (encoding one hydrolase), Km ^R	This study
<i>yqfA</i>	Transposon insertion mutant in gene <i>GLE_1115</i> (encoding one channel protein from hemolysin III family), Km ^R	This study
<i>virB10</i>	Transposon insertion mutant in gene <i>GLE_2820</i> (encoding VirB10 protein), Km ^R	This study
<i>ph</i>	Transposon insertion mutant in gene <i>GLE_5356</i> (encoding one phenol hydroxylase), Km ^R	This study
<i>cysH</i>	Transposon insertion mutant in gene <i>GLE_5487</i> (encoding one phosphoadenosine phosphosulfate reductase), Km ^R	This study
Plasmids		
pBBR1MCS-5	Broad host range vector with a Plac promoter, Gm ^R	(Kovach et al. 1995)
pBBR- <i>dgc</i>	2,228-bp DNA fragment containing the coding region and promoter sequence of <i>dgc</i> (-463 to +1765) (<i>GLE_0067</i>) in pBBR1MCS-5, Gm ^R	This study
pBBR- <i>tetR1</i>	1,475-bp DNA fragment containing the coding region and promoter sequence <i>tetR1</i> (<i>GLE_0809</i>) (-611 to +864) in pBBR1MCS-5, Gm ^R	This study
pBBR- <i>tetR2</i>	1,210-bp DNA fragment containing the coding region and promoter sequence of <i>tetR2</i> (<i>GLE_2933</i>) (-458 to +752) in pBBR1-MCS5, Gm ^R	This study
pBBR- <i>yqfA</i>	1,305-bp DNA fragment containing the coding region and promoter sequence of <i>yqfA</i> (-429 to +876) (<i>GLE_1115</i>) in pBBR1MCS-5, Gm ^R	This study
pBBR- <i>virB8-10</i>	3,301-bp DNA fragment containing the coding region and promoter sequence of <i>virB8-10</i> (-248 to +3053) (<i>GLE_2822</i> , <i>GLE_2821</i> , <i>GLE_2820</i>) in pBBR1MCS-5, Gm ^R	This study
pBBR- <i>ph</i>	1,320-bp DNA fragment containing the coding region and promoter sequence of <i>ph</i> (-373 to +947) (<i>GLE_5356</i>) in pBBR1MCS-5, Gm ^R	This study
pBBR- <i>cysH-hk</i>	3,470-bp DNA fragment containing the coding region and promoter sequence of <i>cysH-hk</i> (-250 to +3220) (<i>GLE_5487</i> , <i>GLE_5488</i>) in pBBR1MCS-5, Gm ^R	This study

^a LeC3, *Lysobacter enzymogenes* strain C3, genome sequence GenBank accession number CP013140.

genomic DNA of overnight bacterial cultures was isolated with a DNA and RNA purification kit, digested with a restriction enzyme (*Pst*I or *Pvu*I), and ligated with T4 DNA ligase. For PCR, DNA samples treated with *Pst*I were amplified with the primer pair KAN-2 FP-1/KAN-2 RP-1, whereas DNA samples treated with *Pvu*I were amplified with the primer pair KAN-2 FP1/*Pvu*I-right or KAN-2 RP1/*Pvu*I-left (Supplementary Table S1). RATE procedure was used as an alternative method to determine the insertion sites for mutants when inverse PCR failed (Clavijo et al. 2006). It is a three-step PCR performed with single primer inv-1 or inv-2, as listed in Supplementary Table S1. The first and third steps used stringent annealing temperature, and the second step used 30°C, which allowed nonspecific amplification (Ducey and Dyer 2002). PCR products from both inverse and RATE PCR were then gel purified and sequenced at the University of Illinois at Urbana-Champaign core sequencing facility. The flanking sequences of the transposon insertion site were analyzed via BLAST search at NCBI. PCR was then performed to confirm the insertion sites with the primers listed in Supplementary Table S1.

Complementation of mutants. A plasmid-based method was used to complement selected mutants as described previously (Qian et al. 2013). Briefly, DNA fragments containing full-length selected genes and their corresponding promoter regions were amplified by PCR with a pair of primers (Supplementary Table S1). Purified PCR products were cloned into pBBR1MCS-5 (Kovach et al. 1995). After confirmed by DNA sequencing with primers M13For-40 and M13Rev-48 (Supplementary Table S1), the final plasmids were electroporated into the corresponding mutants, and the transformants were verified by PCR.

Spore germination assay. *F. virguliforme* Mont-1 isolate was originally obtained from Dr. Glen L. Hartman's laboratory at the University of Illinois. Fungal spores were harvested by washing from PDA plates 7 days postinoculation (DPI) at 28°C. Spore concentration was counted by hemocytometer and adjusted to 2×10^5 per milliliter in 25% potato dextrose broth. Supernatant of 3-day-old bacterial cultures was prepared by centrifugation at 13,200 rpm for 20 min. Equal volumes (1 ml each) of spore

TABLE 2. List of 50 mutants with no inhibition zone in suppressing *Sclerotinia sclerotiorum*^a

Mutant	Insertion site	Accession	Description	Gene
1	73872	GLE_0067	Diguanylate cyclase	<i>dgc</i>
2	182656	GLE_0173/4	NAD-dependent epimerase/dehydratase family protein and hypothetical protein	
3	284302	GLE_0261/2	Hypothetical protein and anhydro-N-acetylmuramic acid kinase	<i>anmK</i>
4	299631	GLE_0270	Peptidase	
5	320871	GLE_0290	Cytochrome c oxidase, subunit III	<i>ccoP</i>
6	384565	GLE_0349	DNA topoisomerase I	<i>topA</i>
7	474196	GLE_0432	Type VI secretion-associated protein, BMA_A0400	<i>tsss</i>
8	480652	GLE_0436	Type VI secretion system Vgr family protein	<i>vgr</i>
9	750857	GLE_0688/9	ATP synthase F1, gamma subunit, and ATP synthase F1, beta subunit	<i>atpG</i> and <i>atpD</i>
10	796945	GLE_0724	Hypothetical protein	
11	870633	GLE_0794	Hypothetical protein	
12	883488	GLE_0809	Transcriptional regulator, TetR family	<i>tetR1</i>
13	995287	GLE_0899	Hypothetical protein	
14	1122532	GLE_1022	Ribosomal protein S6 modification protein	
15	1223320	GLE_1115	Channel protein, hemolysin III family	<i>yqfA</i>
16	1339631	GLE_1223/4	DnaA regulatory inactivator Hda and hypothetical protein	
17	1900985	GLE_1773/4	Rhs family protein and hypothetical protein	
18	2305361	GLE_2122	Transcriptional regulator, ArsR family	
19	2597971	GLE_2373/4	Exported SurF1-family protein and cytochrome o ubiquinol oxidase subunit IV	<i>cyoD</i>
20	2622226	GLE_2396	Transcriptional regulator, Rrf2 family	
21	3187974	GLE_2820	VirB10 protein	<i>virB10</i>
22	3189337	GLE_2821	VirB9 protein	<i>virB9</i>
23	3201951	GLE_2836	Homoserine O-acetyltransferase	<i>metX</i>
24	3238729	GLE_2875	Hypothetical protein	
25	3296438	GLE_2933/4	Transcriptional regulator, TetR family, and hydrolase	<i>tetR2</i>
26	3590009	GLE_3215	HSaf nonribosomal peptide synthetase/polyketide synthase	<i>hsaf pks-nrps</i>
27	3592591	GLE_3215	HSaf nonribosomal peptide synthetase/polyketide synthase	<i>hsaf pks-nrps</i>
28	3996196	GLE_3585	Multiple antibiotic resistance	<i>mar</i>
29	4744813	GLE_4202	Prolyl-tRNA synthetase	<i>proS</i>
30	4833742	GLE_4286/7	Diacylglycerol kinase catalytic domain and RraA family	
31	5066843	GLE_4519	Leukotriene A-4 hydrolase	<i>lta4h</i>
32	5158558	GLE_4600	Hypothetical protein	
33	5505166	GLE_4939/40	Coproporphyrinogen III oxidase, aerobic, and DNA polymerase I	<i>hemF</i> and <i>polA</i>
34	5688480	GLE_5096	Transporter, major facilitator family	
35	5787442	GLE_5193	Glucose-6-phosphate 1-dehydrogenase	<i>zwf</i>
36	5917666	GLE_5315	Hypothetical protein	
37	5942260	GLE_5338/9	DNA polymerase III, beta subunit, and chromosomal replication initiator protein	<i>dnaN</i> and <i>dnaA</i>
38	5960481	GLE_5356	Phenol hydroxylase	<i>ph</i>
39	5995668	GLE_5394	Lipoprotein	
40	6028676	GLE_5430	Lipoprotein	
41	6087030	GLE_5486	Hypothetical protein	
42	6087195	GLE_5486	Hypothetical protein	
43	6088863	GLE_5486	Hypothetical protein	
44	6088893	GLE_5486	Hypothetical protein	
45	6089229	GLE_5486	Hypothetical protein	
46	6089509	GLE_5486/7	Hypothetical protein and phosphoadenosine phosphosulfate reductase	<i>cysH</i>
47	6089556	GLE_5486/7	Hypothetical protein and phosphoadenosine phosphosulfate reductase	<i>cysH</i>
48	6089719	GLE_5487	Phosphoadenosine phosphosulfate reductase	<i>cysH</i>
49	6090763	GLE_5488	Histidine kinase family protein	<i>hk</i>
50	6139312	GLE_5537/8	Hypothetical protein and hypothetical protein	

^a The insertion sites, genes, and annotations are based on the genome sequence of *Lysobacter enzymogenes* strain C3, which can be found in the NCBI with GenBank accession number CP013140.

suspension and bacterial culture supernatant were mixed in 14-ml round-bottom Falcon tubes, vortexed, and then placed in an incubator with shaking at 200 rpm at 25°C. Sixteen hours after cocultivation, 15 µl of each mixture was pipetted onto 25% PDA plates and incubated at 28°C for 3 and half days. Meanwhile, 10 µl of each mixture was pipetted into a hemocytometer and germination examined under a microscope. A spore was considered germinated only if its germ tube was at least the length of the spore. The experiment was repeated twice with three replicates.

Extracellular enzyme activity assays. To determine extracellular activities of protease, cellulase, lipase, and chitinase, 3 µl of bacterial culture adjusted to OD₆₀₀ = 1 was respectively spotted on LB plates containing 1% (mass/vol) skim milk, Van Niel medium (1 g/liter of yeast extract, 0.25 g/liter of MgSO₄, and 20 g/liter of agar) plates containing 1.5% (mass/vol) carboxymethyl cellulose, basal agar medium (10 g/liter of peptone, 15 g/liter of agar, and 0.26 g/liter of CaCl₂ · 2H₂O) plates containing 1% (vol/vol) Tween 80, and 2% (mass/vol) agar plates containing 0.5% (mass/vol) colloidal chitin, at 28°C. For protease activity, diameters of hydrolytic zones surrounding bacterial colonies were measured at one and half DPI. For cellulase activity, plates were stained with 0.1% (mass/vol) Congo red for 30 min at 3 DPI to display hydrolytic zones. For lipase activity, diameters of calcium soap circles surrounding bacterial colonies were measured at 3 DPI. Colloidal chitin used in extracellular chitinase activity assay was prepared from crude chitin as described previously, with minor modifications (Roberts and Selitrennikoff 1988). Briefly, 2.5 g of chitin powder from shrimp shells was completely dissolved in 45 ml of concentrated

HCl and then transferred into 600 ml of water. The mixture was placed at 4°C overnight. Sample pellet was washed several times by removing water on the surface until the pH reached 5.5 to 6.0. After the final wash, colloidal chitin was filtered through three-layer nylon web, autoclaved, and stored at 4°C in dark. The experiments were repeated at least twice with three replications. Fisher's least significant difference procedure was performed via the PROC GLM option of SAS version 9.4 to compare the means of substrate-degrading zone diameters.

HSAF extraction and detection. HSAF extraction was performed as described previously (Qian et al. 2014). Bacterial strains were grown in 50 ml of 10% trypticase soy broth at 28°C for 3 and half days. After centrifugation at 4°C for 30 min, 10 ml of supernatant was collected and vortexed with an equal volume of ethyl acetate. Then ethyl acetate phase containing HSAF was collected and completely dried at room temperature. Final HSAF extraction was resuspended in 200 µl of methanol. An HSAF sample (20 µl) was injected and analyzed with a high-performance liquid chromatography (HPLC) system. The HPLC system included a degasser, an autosampler, a diode array detector, and a quaternary pump. Liquid chromatography separation was performed on a reversed phase HPLC column (4.6 × 150 mm, 5 µm) with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A modified HPLC program was performed as follows: 60 to 100% mobile phase B in mobile phase A from 0 to 10 min, 100% mobile phase B from 10 to 13 min, 100 to 60% mobile phase B in mobile phase A from 13 to 14 min, and 60% mobile phase B in mobile phase A from 14 to 20 min. HSAF was detected at 318 nm with a UV detector. The experiments were repeated twice with similar results.

RESULTS

Identification of Tn5 mutants of LeC3 with reduced suppression of *S. sclerotiorum* hypha growth. To fully understand the molecular mechanism of LeC3 in suppressing fungal hypha growth, random insertion mutant library was screened for mutants lacking in suppressing fungal hypha growth. In total, we obtained 141 mutants with reduced abilities in inhibiting *S. sclerotiorum* hypha growth and determined the Tn5 insertion sites for 50 mutants, which exhibited no inhibition zones in dual culture assays (Table 2). These mutants included mutations in the HSAF biosynthetic gene (*hsaf pks-nrps*) at two locations (i.e., 3590009 and 3592591; #26 and #27) and genes encoding lipoproteins (#39 and #40) and transcriptional factors (the TetR [#12 and #25], ArsR [#18], and Rrf2 [#20] family). Several clustered genes were also found to be mutated, including genes encoding type VI secretion system (#7 and #8), type IV secretion system (T4SS) (#21 and #22), and one region (from #41 to #49) containing a hypothetical gene (*GLE_5486*), *cysH* (*GLE_5487*), and a histidine kinase (*GLE_5488*). These results indicated that these genes or regions may be important in inhibiting *S. sclerotiorum* hypha growth.

Depending on the functions of their predicted proteins based on the standard of Clusters of Orthologous Groups and EggNOG databases (Huerta-Cepas et al. 2015), other identified genes could be grouped into four major classes: (i) cellular processes and signaling, including diguanylate cyclase and multiple antibiotic resistance; (ii) information storage and processing, including DNA topoisomerase, ribosomal protein, and prolyl-tRNA synthetase; (iii) metabolism, including cytochrome c oxidase and phenol hydroxylase, glucose-6-phosphate dehydrogenase, leukotriene A-4 hydrolase, and homoserine O-acetyl; and (iv) functions unknown, including peptidase, hemolysin protein, and transporter from major facilitator family (Table 2). In addition, genes for 15 mutants encode hypothetical proteins without putative functions.

For the rest of this study, we selected seven mutants for further characterization, including genes encoding TetR family transcriptional regulators (*tetR1* and *tetR2*), diguanylate cyclase (*dgc*),

Fig. 1. Effect of *Lysobacter enzymogenes* mutants in inhibiting *Sclerotinia sclerotiorum* hypha growth. **A**, The *dgc* mutant (left) and complementation (right); **B**, the *tetR1* and *C*, *tetR2* mutant (left) and complementation (right); **D**, the *yqfA* (left) and complementation (right); **E**, the *virB10* (left) and complementation (right); **F**, the *ph* (left) and complementation (right); and **G**, the *cysH* (left) and complementation (right). On 10% trypticase soy agar (A and E) or 25% potato dextrose agar (B to D, F, G) plates, 5 µl of wild-type *L. enzymogenes* strain C3 (top), mutant (left), and corresponding complementation strain (right) with OD₆₀₀ = 0.2 were cocultured with *S. sclerotiorum*, except for D, 12.5 µl with OD₆₀₀ = 0.4 and G, 100 µl with OD₆₀₀ = 0.5.

hemolysin III family channel protein (*yqfA*), type IV secretion system VirB10 protein (T4SS, *virB10*), phenol hydroxylase (*ph*), and phosphoadenosine phosphosulfate reductase (*cysH*) (Table 1). First, we showed that complementation of the seven mutants partially restored their abilities in inhibiting *S. sclerotiorum* hypha growth. In a dual culture assay with *S. sclerotiorum*, clear inhibition

zones were observed around LeC3 (Fig. 1A to G, top), whereas no inhibition zones were seen around the seven mutants (Fig. 1A to G, left). For complementation strains, complete or partial recovery of inhibition zones was observed, some with very clean zones (*dgc* and *virB10*, Fig. 1A and E, right), whereas others with fewer visible hyphae (Fig. 1B to D, F and G, right). These results indicate that

Fig. 2. *Lysobacter enzymogenes* mutants exhibited decreased abilities in inhibiting *Fusarium virguliforme* **A**, spore germination and **B**, growth. Supernatants of 3-day-old bacterial culture were mixed with 2×10^5 conidia spore/ml of *F. virguliforme* overnight in a shaking incubator (200 rpm) at 25°C. Spore germination was examined under microscope in hemocytometer, and 15 μ l of each overnight mixture was pipetted onto 25% potato dextrose agar plates and incubated at 28°C. Pictures were taken 3.5 days after inoculation. *cysH*, the *cysH* mutant; *dgc*, the *dgc* mutant; *ph*, the *ph* mutant; *tetR1* and *tetR2*, the *tetR1* and *tetR2* mutants; *virB10*, the *virB10* mutant; WT, wild type *L. enzymogenes* strain C3; *yqfA*, the *yqfA* mutant.

these genes are responsible for inhibiting *S. sclerotiorum* hypha growth.

Mutations of the seven genes in *L. enzymogenes* also led to decreased abilities in inhibiting *F. virguliforme* spore germination and growth. Under microscope, *Fusarium* spores mixed with LeC3 supernatant were either lysed or not germinated, making the germination rate 0, whereas germination rate for blank control was almost 1. In contrast, *Fusarium* spores mixed with supernatants of the seven mutants exhibited higher germination rates, ranging from 0.02 to 0.74 (Fig. 2A). Complementation of the seven mutants restored spore germination rate to almost 0 (Fig. 2A). In addition, growth of *Fusarium* spores mixed with LeC3 was severely inhibited as compared with control (Fig. 2B). Spores mixed with seven mutants grew much faster than that of LeC3, indicating that the seven mutants also decreased in their abilities in inhibiting *F. virguliforme* spore growth (Fig. 2B), whereas *Fusarium* spores mixed with complementation strains exhibited less growth (Fig. 2B). These results suggested that these seven genes are also responsible for inhibiting *F. virguliforme* spore germination and growth.

Extracellular enzyme activities were decreased in the seven mutants. To determine what contributes to the reduced activities against fungal pathogens, extracellular enzyme activities of the seven mutants were determined. Protease, cellulase, and lipase activities were significantly lower in the seven mutants than those of LeC3 (Figs. 3A to C and 4A to C). Significant differences were also observed among the seven mutants, where the *cysH* mutant exhibited the lowest activities. Furthermore, no hydrolytic

zones around the *dgc*, *virB10*, and *cysH* mutants were observed on 2% agar plates containing 0.5% colloidal chitin, suggesting that these mutants no longer had detectable chitinase activities (Figs. 3D and 4D). Chitinase activities of the other mutants were significantly lower than that of LeC3, whereas significant differences in chitinase activities were also observed among the seven mutants, except *tetR1* mutant (Figs. 3D and 4D).

Extracellular enzyme activities in complementation strains were partially restored for most mutants, as shown in Figure 4. As an example, complementation of the *virB10* mutant showed significantly higher activity of cellulase and chitinase (Fig. 5A). Complementation of the *tetR1*, *yqfA*, and *cysH* mutants exhibited significantly increased extracellular protease activities (Fig. 5B). Moreover, complementation of the *yqfA*, *virB10* (in LB containing 0.5% Tween 80), and *cysH* mutants exhibited significantly increased extracellular lipase activity (Fig. 5C). However, most complementation strains exhibited slightly higher enzyme activities than those of their corresponding mutants (Fig. 4). These results showed that the seven genes play a role in extracellular enzyme activities, which might contribute to antifungal activities.

HSAF was not detectable in the seven mutants. To test whether the seven genes contribute to HSAF biosynthesis, their HSAF production was determined by HPLC. As shown in Figure 6, the single HSAF peak was detected in the sample of HSAF standard and LeC3, whereas no HSAF peak was detected in the *hsafpks-nrps* mutant, which was previously proven to be defective in HSAF production and serve as a negative control (Fig. 6). Interestingly,

Fig. 3. Effect of mutations on extracellular enzyme activities. **A**, Protease; **B**, cellulase; **C**, lipase; and **D**, chitinase. Protease, cellulase, lipase, and chitinase activity was determined on Luria-Bertani plates supplemented with 1% skim milk, Van Niel medium containing 1.5% carboxymethyl cellulose, basal agar medium containing 1% Tween 80, and 2% agar medium containing 0.5% colloidal chitin, respectively. Extracellular enzyme activities were determined from the diameter of hydrolytic zones (calcium soap zone in lipase case) around bacterial colonies on testing plates. Different letters indicate significant differences from one-way analysis of variance followed by Fisher's least significant difference test ($P < 0.05$). *cysH*, the *cysH* mutant; *dgc*, the *dgc* mutant; *ph*, the *ph* mutant; *tetR1* and *tetR2*, the *tetR1* and *tetR2* mutants; *virB10*, the *virB10* mutant; WT, wild type *L. enzymogenes* strain C3; *yqfA*, the *yqfA* mutant.

samples from all seven mutants showed no detectable HSAF. Despite multiple attempts and scale-up sample preparations, HSAF was not detectable in all seven complementation strains (data not shown). Although our results were not conclusive, these results nonetheless suggested that the seven genes might be involved in regulation of HSAF biosynthesis or secretion of HSAF in *L. enzymogenes*.

DISCUSSION

L. enzymogenes strains have been considered as potential BCAs, including strains N4-7, LeC3, 3.1T8, and OH11 (Folman et al. 2003; Giesler and Yuen 1998; Kobayashi and Nour 1996; Qian et al. 2009). Understanding of their interactions with fungi or other pathogens is far from complete. In this study, we obtained 50 mutants of LeC3 with no inhibition of *S. sclerotiorum* hypha growth, and the insertion sites for about one fourth of these mutants were located within three 4-Kb regions, including the T4SS gene cluster, the HSAF biosynthesis gene *hsafpks-nrps*, and a three-gene operon, which encodes a hypothetical protein, a phosphoadenosine phosphosulfate (PAPS) reductase, and a histidine kinase family protein. These results indicated that these regions might be important in its inhibitory activities against the white mold pathogen *S. sclerotiorum* (Clavijo et al. 2006; Ducey and Dyer 2002; Xia et al. 2015). Previous studies revealed that mutation in the *hsafpks-nrps* gene no longer inhibited fungal hypha growth, which is consistent with our results (Yu et al. 2006). It is now known that the hybrid polyketide synthase and nonribosomal peptide synthetase encoded by the *hsafpks-nrps* gene catalyzes the essential step of linking ornithine to polyketides in the biosynthesis of HSAF (Lou et al. 2011). Further studies also showed that small metabolites, 4-hydroxybenzoic acid and 13-methyltetradecanoic acid, could increase the transcription of the *hsafpks-nrps* gene, leading to increased HSAF production (Han et al. 2015; Su et al. 2017).

T4SSs in Gram-negative bacteria are found to have versatile functions, including conjugative transformation, mediation of genetic exchange, and delivery of macromolecular effectors (such as toxin and T-DNA) into eukaryotic cells (Fronzes et al. 2009). The Type IVA T4SSs (VirB-VirD4 conjugative system) consist of 12 proteins, i.e., VirD4 and VirB1 to VirB11. The periplasmic lytic transglycosylase VirB1 is required for pilus biogenesis and degradation of peptidoglycan layer; VirB2 and BirB5 is responsible for the formation of pilus which will extend into extracellular milieu; proteins VirB3 and VirB6 to VirB10 collectively form the scaffold and translocation apparatus; whereas VirB4, VirB11, and VirD4 are three ATPases which power T4SSs (Costa et al. 2015). The LeC3 genome harbors a *virB1-11* cluster with a distantly located *virD4* gene, as well as two copies of the *virB5* gene and multiple pairs of the *virB5-virB6* gene cluster (de Bruijn et al. 2015). In this study, we identified two mutants (*virB10* and *virB9*) located within the T4SS cluster, suggesting that T4SS in LeC3 might play an important role in suppressing fungal hypha growth. Although extracellular enzymes are normally secreted by type II secretion system, we found that the *virB10* mutant exhibited significantly reduced protease, cellulase and lipase activities as well as no chitinase activity.

Insertion sites of eight mutants were within a three-gene operon, which encodes a hypothetical protein, a PAPS reductase, and a histidine kinase family protein. In *E. coli*, the PAPS reductase catalyzes the conversion of PAPS into sulfite and adenosine 3',5'-bisphosphate (PAP) in the sulfate reduction pathway, which leads to the assimilation of sulfur and biosynthesis of cysteine (Rossi et al. 2014). Recent studies have revealed that other intermediates in *E. coli* sulfate assimilation and cysteine biosynthesis pathway (e.g., PAP, PAPS) can also act as a global signal molecule and modulate gene expression (Longo et al. 2016). Inactivation of the *cysH* gene increased intracellular PAPS concentration and consequently increased cell aggregation and surface adhesion by regulation over the expression of curli components, including Flu, OmpX, Slp, and

other regulators CspC, CspE, HNS, and HupA (Longo et al. 2016). Because the PAPS reductase in *E. coli* and *L. enzymogenes* shares 66% deduced amino acid similarity, and immediately after the *cysH* gene is a gene encoding a signal transduction histidine kinase in LeC3, it is tempting to speculate that loss of inhibition of fungal hypha growth and spore germination may be caused by affecting the PAPS-mediated regulatory system in *L. enzymogenes*.

In *Xanthomonas campestris*, it has been proven that the sensor kinase RpfC detects the diffusible signal factor signal and activates the response regulator RpfG, which degrades c-di-GMP and thus activates virulence genes (Tao et al. 2010). In addition, c-di-GMP specifically interacts with the global regulator Clp, leads to the allosteric conformational changes of Clp, and abolishes binding to

Fig. 4. Extracellular enzyme activities of *Lysobacter enzymogenes* strain C3, mutants, and their complementation strains. **A**, Protease; **B**, cellulase; **C**, lipase; and **D**, chitinase. Protease, cellulase, lipase, and chitinase activity was determined on Luria-Bertani plates supplemented with 1% skim milk, Van Niel medium containing 1.5% carboxymethyl cellulose, basal agar medium containing 1% Tween 80, and 2% agar medium containing 0.5% colloidal chitin, respectively. *cysH*, the *cysH* mutant; *dgc*, the *dgc* mutant; *ph*, the *ph* mutant; *tetR1* and *tetR2*, the *tetR1* and *tetR2* mutants; *virB10*, the *virB10* mutant; WT, wild type *L. enzymogenes* strain C3; *yqfA*, the *yqfA* mutant.

its target gene promoter (Tao et al. 2010). Considering the similarity of the *clp* and *rpf* gene cluster between *X. campestris* and *L. enzymogenes*, c-di-GMP signaling has been reported to play an important role in *L. enzymogenes* (Chen et al. 2017). In *L. enzymogenes*, the intracellular concentration of c-di-GMP

increased in the *pilG* mutant, which was defective in HSAF production (Chen et al. 2017). The diguanylate cyclases synthesize c-di-GMP, whereas the phosphodiesterases degrade c-di-GMP (Jenal and Malone 2006). Interestingly, mutation of a diguanylate cyclase (*dgc*) gene resulted in loss of antifungal activity and HSAF

Fig. 5. Complementation of *Lysobacter enzymogenes* mutants in extracellular enzyme activities. **A**, Cellulase and chitinase activities were determined on Van Niel medium containing 1.5% carboxymethyl cellulose (left) and 2% agar medium containing 0.5% colloidal chitin (right), respectively. **B**, Protease activity was determined on Luria-Bertani (LB) plates supplemented with 1% skim milk. **C**, Lipase activity was determined on basal agar medium containing 1% Tween 80 for *yqfA* and *cysH* and on LB medium containing 0.5% (vol/vol) Tween 80 for *virB10*. Extracellular enzyme activities were determined from the diameter of hydrolytic/calcium soap zones around bacterial colonies on testing plates. Different letters indicate significant differences from one-way analysis of variance followed by Fisher's least significant difference test ($P < 0.05$). *cysH*, the *cysH* mutant; *dgc*, the *dgc* mutant; *ph*, the *ph* mutant; *tetR1* and *tetR2*, the *tetR1* and *tetR2* mutants; *virB10*, the *virB10* mutant; WT, wild type *L. enzymogenes* strain C3; *yqfA*, the *yqfA* mutant.

production, as well as reduced extracellular enzyme activities. These results suggest that c-di-GMP played a positive role in HSAF biosynthesis and extracellular enzyme production.

Survival of bacteria under various environments requires a multitude of rapid and adaptive responses triggered by regulatory proteins, which in most cases are transcriptional factors (TFs) (Ramos et al. 2005). Previous studies showed that LetR, a TetR-family TF in *L. enzymogenes*, bound to the promoter of HSAF biosynthesis operon and suppressed HSAF biosynthesis (Ramos et al. 2005; Wang et al. 2017). In this study, we identified two TetR family TFs, and both the *tetR1* and *tetR2* mutants showed decreased HSAF production, suggesting a positive effect on HSAF production. It is possible that different TetR family TFs may play different roles in regulation of HSAF production.

Phenol hydroxylase is predicted to be a ferredoxin-NADP reductase. This reductase is a flavin adenine dinucleotide-containing enzyme that catalyzes the reversible electron transfer between NADPH and electron carrier proteins (i.e., ferredoxin and flavodoxin) (Nogués et al. 2005). The C-terminal domain contains most of the NADPH binding residues, whereas the N-terminal domain interacts noncovalently with the isoalloxazine rings of the flavin molecule. Ferredoxin-NADP⁺ reductase first accepts one electron from reduced ferredoxin to form a flavin semiquinone intermediate. The enzyme then accepts a second electron to form FADH₂, which then transfers two electrons and a proton to NADP⁺ to form NADPH. Isoforms of these flavoproteins in bacteria participate in a wide variety of redox metabolic pathways (Nogués et al. 2005). In this study, deletion of the *ph* gene led to loss of activity against *S. sclerotiorum* and *F. virguliforme*, reducing extracellular enzyme activities and HSAF production. These results suggest that changes in redox-related metabolic pathways might lead to the loss of biocontrol activities in LeC3.

Members of the hemolysin III family are integral membrane proteins. In *Bacillus cereus*, hemolysis resulting in 3- to 3.5-nm hemolytic pores occurred in at least three steps: binding of Hyl-III monomers to the erythrocyte membrane, formation of transmembrane oligomeric pore, and erythrocyte lysis (Baida and Kuzmin 1996). The channel forming protein YqfA is predicted to participate in intracellular trafficking, secretion, and vesicular transport. Outer membrane vesicles (OMVs) produced by LeC3

were discovered to contain chitinase and HSAF activities, suggesting that OMVs acted as remote mobile component of predation by LeC3 (Meers et al. 2018). This finding is consistent with our results that loss of YqfA function led to loss of extracellular enzyme and HSAF activities. It is reasonable to predict that YqfA affected biocontrol activity through its influence on OMV production, which warrants further investigation.

In summary, we identified and characterized several novel genes in suppressing soybean fungal hypha growth and spore germination and provided new information about the complex interaction between LeC3 and soybean pathogens at the molecular level. However, detailed characterization of these genes and understanding of their exact roles in extracellular enzymes and HSAF production and secretion in *L. enzymogenes* are needed.

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Fig. 6. Production of heat-stable antifungal factor (HSAF) in *Lysobacter enzymogenes* and mutant strains. Standard HSAF, *Lysobacter enzymogenes* strain C3 (LeC3), and the HSAF-deficient *hsaf pks-nrps* mutant were used as positive and negative controls. The single HSAF peak (marked by arrows) was detected by high-performance liquid chromatography in HSAF standard and LeC3 but not in the *hsaf pks-nrps* mutant or the seven selected mutants. *cysH*, the *cysH* mutant; *dgc*, the *dgc* mutant; *ph*, the *ph* mutant; *tetR1* and *tetR2*, the *tetR1* and *tetR2* mutants; *virB10*, the *virB10* mutant; WT, wild type *L. enzymogenes* strain C3; *yqfA*, the *yqfA* mutant.

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