Insight Into Genes Involved in the Production of Extracellular Chitinase in a Biocontrol Bacterium *Lysobacter enzymogenes* C-3

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The chitinase producing *Lysobacter enzymogenes* C-3 has previously been shown to suppress plant pathogens *in vitro* and in the field, but little is known of the regulation of chitinase production, or its role in antimicrobial activity and biocontrol. In this study, we isolated and characterized chitinase-defective mutants by screening the transposon mutants of *L. enzymogenes* C-3. These mutations disrupted genes involved in diverse functions: glucose-galactose transpoter (*gluP*), disulfide bond formation protein B (*dsbB*), Clp protease (*clp*), and polyamine synthase (*speD*). The chitinase production of the *SpeD* mutant was restored by the addition of exogenous spermidine or spermine to the bacterial cultures. The *speD* and *clp* mutants lost *in vitro* antifungal activities against plant fungal pathogens. However, the *gluP* and *dsbB* mutants showed similar antifungal activities to that of the wild-type. The growth of the mutants in nutrient rich conditions containing chitin was similar with that of the wild-type. However, growth of the *speD* and *gluP* mutants was defective in chitin minimal medium, but was observed no growth retardation in the *clp* and *dsbB* mutant on chitin minimal medium. In this study, we identified the four genes might be involved and play different role in the production of extracellular chitinase and antifungal activity in *L. enzymogenes* C-3.

Keywords : biological control, chitinase, polyamine, transposon mutagenesis

Certain bacterial strains inhibit the growth of plant pathogens by production of various antimicrobial compounds and extracellular hydrolytic enzymes (Kim et al., 2011). Chitinase-producing microbes have been extensively used as commercial biocontrol agents as the extracellular chitinase

enzyme hydrolyzes the β -1,4-linkage of chitin, which is widely distributed the cell walls of plant pathogenic fungi (Flach et al., 1992; Kim et al., 2008). It has been proposed that extracellular chitinase is involved in the biological control of plant pathogens by biocontrol bacterial species of the genera of *Bacillus* (Chang et al., 2003; Lee et al., 2009; Reyes-Ramirez et al., 2004) and the fungal species of the genus *Trichoderma* (Lorito et al., 1993). Chitinase production by *Serratia plymuthica* was not found to be important for its biocontrol (Kamensky et al., 2003), but *in vitro* antifungal activities against *Rhizoctonia solani* of *Chromobacterium* sp. C61 and a *Pseudomonas* sp. GRC3 were related to the production of extracellular chitinase (Arora et al., 2007; Park et al., 2005). Because plant disease suppression results from multiple activities of effective biocontrol bacteria, it is important to determine which activities contribute the most to biological control under various conditions (Kim et al., 2011). The role of chitinase in the biological control of plant pathogens was mainly supported from studies showing that chitinolytic bacteria or their purified enzymes inhibit fungal growth *in vitro* and *in vivo* (Chet et al., 1990; Lorito et al., 1994).

The regulatory network for the expression of chitinase in bacteria is largely unknown. A *clp* protease is known to be a global regulator for the expression of extracellular chitinase in *Lysobacter enzymogenes* C3 (Kobayashi et al., 2005). A GacS/GacA two component regulatory component and ChiR, a LysR-type transcriptional regulator, are proposed as global regulators for the production of chitinase in *Serratia plymuthica* (Ovadis et al., 2004; Suzuki et al., 2001). Recent studies indicated that the small RNA regulator *rsmA* in *Listeria monocytogenes* (Nielsen et al., 2011), and a novel sigma factor-like RpoQ in *Vibrio fischeri* (Cao et al., 2012), were found to be global regulators of the chitinase production.

A chitinolytic bacterium, *Lysobacter enzymogenes* C-3 was isolated from soil around a pear orchard in Korea and was used as an effective bioformulated product with other chitinase producing microbes (Kim et al., 2008). Strain C-3

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showed a broad spectrum of antifungal activity to various plant pathogenic fungi and was used in the control of Phytophthora blight in pepper, and Alternaria blight and anthracnose in Ginseng (Kim et al., 2008; 2010). However, bacterial traits related biocontrol properties, such as chitinase and antifungal activity, and their contribution in biocontrol and regulatory components on expression of chitinase are largely unknown in *L. enzymogenes* C-3. In this work, our primary goal was to isolate and characterize the mutants which are reduced in production of chitinase in *L. enzymogenes* C-3 using a transposon mutagenesis approach.

A random transposon mutant library of *L. enzymogenes* C-3 was constructed using an EZ-Tn5 DHFR-1 Tnp transposome kit as described by the manufacturer (Epicentre, Madison, USA). Four transposon mutants displaying absent or reduced clear zones on chitin agar plates were obtained from *in vitro* screening of 2,560 mutants. When the bacterial strains were incubated for 6 days at 28 °C on chitin minimal medium (Kim et al., 2008), a clear zone of strain C-3 was detected, but three transposon mutants (M1, M4, and M10) did not show any clear zone, and mutant M17 showed a reduced clear zone on chitin minimal medium (Fig. 1A).

In order to identify the genes involved in the production of chitinase in C-3 at the genetic level, genome DNAs from logarithmic cells of wild-type and the four mutants of C-3 grown in nutrient broth (NB) were isolated using CTAB-NaCl method (Ausubel et al., 1989). The Tn5 flanking sequences of the mutants were determined using DNA Walking Speedup Kit I (SeeGene Inc., Seoul, South Korea) with Annealing Control Primers (ACP) and the EZ-Tn transposon specific DHFR primers as described in the

manufacturer's protocol. Three polymerase chain reactions (PCR) with different primer sets in the kit were conducted to amplify the specific transposon flanking sequences in the mutants. The primers were as follows: for DNA Walking-Annealing Control Primer 1 (DW-ACP1) for first PCR, 5'-ACP-AGGTC-3'; DW-ACPN primer for second PCR, 5'-ACPN-GGTC-3', ACP universal primer for third PCR, 5'-TCA CAG AAG TAT GCC AAG CGA-3'; and target specific primers for the transposon, DHFR-1 FP-1 forward primer 5'-GGC GGA AAC ATT GGA TGC GG-3' or reverser primer 5'-GAC ACT CTG TTA TTA CAA ATC G-3'). The PCR reaction was conducted with pre-mix provided by SeeGene Inc. (Seoul, South Korea) in the GenAmp 2400 thermal cycler (Perkin Elmer, San Jose, USA). PCR products were cleaned using QIAquick columns (Qiagen Inc., Valencia, USA) and were cloned with the pGEM-T Easy kit (Promega, Inc., Madison, USA). Recombinant plasmids containing PCR inserts were isolated using the mini-plasmid purification system (Bioneer Inc., Daejeon, Korea). The inserts were sequenced using dye terminator on an ABI1301 DNA sequencer (Applied Biosystems, Foster City, USA) at Solgent Company (Solgent Inc., Daejeon, South Korea). The obtained sequences were identified with the GenBank nucleotide sequence database through a BLAST search. All of the four mutants possessed single sites of insertion of the EZ-Tn5 transposon (data not shown).

Analysis of the Tn5 flanking sequences of the mutants showed that the transposon inserted in different genes in each mutant. Table 1 shows the site of the mutations and the proposed functions of the open reading frame of the inactivated genes. Two of the mutants M10 and M17 had the insertion sites in adjacent genes in the open reading

Fig. 1. Clearing of colloidal chitin (A) and inhibition zones against *Collectotrichum acutatum* and *Fusarium oxysporum* (B) induced by growth of colonies of *Lysobacter enzymogenes* strain C-3 and its mutants. Clearing zones were observed 6 days after inoculation of the bacterium on medium containing colloidal chitin and inhibition of fungal growth was observed 4 days after inoculation of potato dextrose agar with bacteria and fungal pathogen.

Table 1. Predicted functions of the Tn5-flanking sequences of the *Lysobacter enzymogenes* C-3 chitinase defective mutants

Mutants	Highest homology sequence of the Tn5 inserted CDSs (Genbank accession number)*	Putative function or description
M1	Between 368 E and 369 T amino acid sequence in a <i>gluP</i> gene product of <i>Pseudoxanthomonas suwonensis</i> 11-1 (CP002446.1)	Glucose/galactose transporter
M4	Between 102 P and 103 E amino acid sequence in a <i>dspB</i> gene product of <i>Pseudoxanthomonas spadix</i> BC-a59 (CP003093.2)	Disulfide bond formation protein B
M10	Between 179 R and 180 K amino acid sequence in a <i>speD</i> gene product of <i>Lysobacter enzymogenes</i> C3 (AY316743.1)	S-adenosylmethionine decarboxylase
M17	Between 81 G and 82 E amino acid sequence in a <i>clp</i> gene product of <i>Lysobacter enzymogenes</i> C3 (AY316743.1)	CAP-like protein, Clp regulator

*Insertion sites were identified based on comparison of the deduced amino acid sequences of the flanking regions in the mutants to the deduced amino acid sequences of the represented genes which showed the highest homology by the blast search.

frames for a protein encoding S-adenosylmethionine decarboxylase (*speD*) and Clp regulator (*clp*), respectively. Other insertion sites included: mutant M1 insertion in *gluP* encoded for glucose/galactose transporter, and mutant M4 in open reading frame of *dspB* gene encoding a disulfide bond formation protein B (Table 1).

In previous work of other group, a similar approach was used to identify a global regulator of chitinase production, and the Clp protease was identified as a global regulator of expression of extracellular chitinase and biocontrol traits in *L. enzymogenes* (Kobayashi et al., 2005). Interestingly, a transposon inserted in the *speD* gene, which is located to juxtaposition of the *clp* gene in the mutant M10, showed similar phenotypes in the production of extracellular lytic enzyme.

The S-adenosylmethionine decarboxylase in the M10 mutant is involved in biosynthesis of a polyamine, spermidine (Shah and Swiatlo, 2008). Spermidine is synthesized by the conversion of methionine into S-adenosylmethionine (SAM) by the enzyme SAM synthase, followed by the conversion of SAM into decarboxylated SAM by the enzyme SAM decarboxylase (SpeD). The spermidine synthase (SpeE) converts putrescine to spermidine in the presence of decarboxylated SAM (Shah and Swiatlo, 2008). In *E. coli*, polyamines were not required for aerobic growth, but were important for anaerobic growth by the mutational analysis of the polyamines (Chattopadhyah et al., 2009). In human pathogenic bacteria, polyamines (putrescine and spermidine) are important for production of virulence factors (Jelsbak et al., 2012; Shah and Siatlo, 2008). *Salmonella enterica* polyamine mutant was showed defective in production of virulence factors, such as invasion, intracellular survival, killing of the nematode, and systemic infection of the mouse. The defective phenotypes of the *S. enterica* polyamine mutant were restored by the addition of exogenous putrescine and spermidine (Jelsbak et al., 2012). In this study, the production of extracellular chitinase in the

mutants of M10 was recovered to the levels of the wild-type when an external polyamine was amended in the chitin minimal medium (Fig. 2). These results indicated that internal polyamine(s) in the SpeD mutant might play an important role in the production of extracellular chitinase in *L. enzymogenes* C-3.

Previous studies have shown that disulfide bond forming protein B (DsbB) is a membrane component of the pathway that leads to disulfide bond formation in periplasmic proteins of *Escherichia coli* (Bardwell et al., 1997), and re-oxidizes the periplasmic protein, DsbA, which is a key periplasmic protein in forming disulfide bonds in exported proteins (Heras et al., 2009). The glucose/galactose transporter is known as a membrane protein involved in glucose or galactose transport in *Brucella abortus* and is a member of the major facilitator superfamily (Essenberg et al., 1997).

The reduction of chitinase production in the mutants

Spermidine (50 µg/ml)**Spermine (50 µg/ml)**

Fig. 2. Complementation of the chitinase deficient phenotypes of the *Lysobacter enzymogenes* mutant M10 (SpeD) by the addition of polyamines. Extracellular chitinase production of the *L. enzymogenes* mutant 10 (SpeD) was determined by observation of clearing colloidal chitin minimal medium containing 50 µg/ml of spermidine or spermine. Photo-images of the clearing zones were taken 6 days after inoculation of bacterium on chitin minimal medium containing the exogenous polyamine.

might result from defects in their growth on chitin as the sole carbon source. To test this possibility, we determined the effect of amended chitin on growth and production of chitinase of the chitinase deficient mutants under nutrient rich and minimal growth conditions. *L. enzymogenes* C-3 bacterial cells were grown in nutrient broth at room temperature with shaking for 36 h, harvested by centrifugation and re-suspended with sterile water. After adjust OD_{600 nm} = 0.1 with sterile water, bacterial cells were inoculated to 1/2 Luria Bertani (LB) or chitin minimal medium (Kim et al., 2008) containing 0.2% colloidal chitin at 28°C for 48 h with agitation. At defined times, viable cells of each bacterial strain were serially diluted and counted on LB agar plates containing appropriate antibiotics. In addition, chitinase activity was assessed in the culture supernatant after centrifugation as described previously (Kim et al., 2008). Enzyme activity was assayed by measuring hydro-

lysis of 4-methylumbelliferyl- β -D-*N,N*-diacetylchitobioside (Sigma, St. Louis, MO) in a reaction mixture (100 μ l) containing 0.1 mM substrate and enzyme in 100 mM KH₂PO₄ buffer pH 7.0. After incubation for 10 min at 37°C, the reaction was stopped by addition of 0.1 ml of 0.2 M Na₂CO₃, and chromogen formation was measured spectrometrically with excitation at 360 nm and emission at 440 nm in a fluorometer, Bio-Tek FLX-8000 (Bio-Tek Inc., Winooski, USA). One unit of chitinase activity was expressed as micromoles of liberated 4-methylumbelliferone per 1 h per milliliter of culture supernatant. This experiment was repeated independently three times.

The growth and chitinase production of the *L. enzymogenes* C-3 chitinase-deficient mutants differed between the nutrient rich and minimal growth conditions containing chitin as a sole carbon source. Wild-type C-3 strain normally showed extracellular chitinase activity in the medium

Fig. 3. Production of extracellular chitinase and cell growth of *Lysobacter enzymogenes* C-3 and its mutants under 1/2 LB broth and minimal medium broth containing chitin. Bacterial cells were grown in nutrient broth at room temperature with shaking for 36 h, harvested by centrifugation and re-suspended with sterile water. After adjust OD_{600nm} = 0.1 with sterile water, bacterial cells were inoculated to the indicated growth medium and were incubated with shaking at room temperature. At various stages after inoculation, the extracellular chitinase activity in the cell free culture supernatant was measured with 4-MU-(GlcNAc)₂ in the phosphate buffer (pH 7.0) as described in the text. The enzyme activity (U/ml culture supernatant) was expressed as mM of liberated 4-methylumbelliferone per 1 h. At various stages after inoculation, the viable cell density was measured by spreading of bacterial cultures (serially diluted 10-fold) on nutrient agar plates. Each value represents the mean of three independent experiments, and the same letter represents no significance according to Duncan's multiple range test ($P < 0.05$).

containing chitin (Table 2), but did not produced chitinase in the growth medium without chitin (data not shown). On the other hand, no detectable level of the chitinase activity was observed in the cell-free culture filtrate of the M1 or M10 mutants grown in any of the examined media (Fig. 3). The extracellular chitinase activity of the mutant M17 was significantly reduced in both media to compare to levels of the wild-type strain. Meanwhile, significantly reduced level of chitinase activity was detected in the mutant M4 grown in chitin minimal medium, and no chitinase activity was detected in the 1/2 LB chitin medium (Fig. 3).

The growth of the chitinase-deficient mutants, M1 and M10, was reduced in chitin minimal medium (Fig. 3), but they grew better than wild-type in 1/2 LB chitin growth condition. However, the mutants of M4 and M17 grew in a similar manner to the wild-type in both growth media (Fig. 3). These results indicated that the chitinase deficiencies of *gluP* (M1) and *speD* (M10) mutants under chitin as a sole carbon source might result from auxotrophic phenotypes of the mutants. The auxotrophic phenotypes of the glucose/galactose transporter mutant and *SpeD* mutant under minimal medium growth condition might be a main reason for chitinase deficiency on chitin minimal medium. However, the disulfide bond formation B and Clp regulator play important role in the production of extracellular chitinase in *L. enzymogenes* C-3. The Dsb components in the periplasm of bacteria have been proposed to play important role in the secretion of virulence factors, such as adhesion, toxin production, secretion of extracellular enzymes and motility, in various bacteria (Dutton et al., 2008; Heras et al., 2009). We hypothesized that the extracellular chitinase enzyme(s) might be secreted through the Dsb pathway in the periplasm, and that the Dsb components are required for active extracellular chitinase formation in *L. enzymogenes* C-3 as described for *E. coli* (Dutton et al., 2008; Heras et al., 2009). To test our hypothesis, we are currently constructing Dsb mutants.

To determine role of chitinase in growth inhibition of plant pathogenic fungi, we measured antifungal activity of the mutants. *L. enzymogenes* C-3 wild-type and the chitinase defective mutants grown in agar plates were harvested, re-suspended, and adjusted to OD_{600nm} = 1.0 with sterile water. Plant pathogenic fungi were obtained from the Korean Agriculture Culture Collection Center (KACC, Suwon, South Korea), and were grown on potato dextrose agar (PDA) at 28°C. Agar disks (0.5 cm in diameter) of *Fusarium oxysporum* and *Collectotrichum acutatum* grown on PDA for 7 days at 28°C were placed in the center of the plate, and suspensions of the *L. enzymogenes* C-3 wild type and the chitinase defective mutants were spotted onto PDA. After incubation at 28°C for 4 days, the inhibition zone between the edges of the bacterial colony and the fungal

mycelium was observed. Inhibition of hyphal growth of *F. oxysporum* and *C. acutatum* on PDA was inhibited greatly in presence of wild type C-3, M1 and M4 mutants, but no inhibition was detected with M10 and M17 mutants (Fig. 1B). These results indicated that the production of extracellular chitinase and antifungal activity in *L. enzymogenes* C-3 were not directly correlated. Previous work in *L. enzymogenes* C3 showed that reduction of production of extracellular chitinase, protease, and antimicrobial activity (Kobayashi et al., 2005). In this study, mutant 17 (Clp) showed similar phenotypes as the Clp mutant of C3. In addition to reduction of chitinase production, production of extracellular protease in the mutant M17 was significantly reduced, about 50% smaller clear zone in size compared to that of wild type on 1% skim milk agar plate, and deficiency of antifungal activity (Fig. 1). These results indicated that Clp could be a global regulator for expression of production of extracellular enzymes and antimicrobial activity.

Chitinolytic enzymes produced by bacteria and fungi have been postulated to play an important role in the biocontrol of fungal pathogens (Chang et al., 2003; Kobayashi et al., 2002; Lee et al., 2009; Lorito et al., 1993; Reyes-Ramirez et al., 2004). Chitinases are divided into two different families (18 and 19) based on amino acid sequence similarities (Henrissat and Davies, 1997). Plant chitinases and some bacterial chitinases from *Streptomyces* species are grouped in the family of 19 chitinases which are known to have strong antifungal activities (Cohen-Kupiec and Chet, 1998; Ohno et al., 1996). Most bacterial chitinases, including most of the Gram negative bacteria and *Bacillus* species belong to family 18. Gram positive bacterial strains, *Bacillus thuringiensis* and *B. cereus*, of the family 18 chitinases were shown to produce an important extracellular enzyme in the biocontrol of plant fungal diseases (Chang et al., 2003; Li et al., 2008; Reyes-Ramirez et al., 2004). One recent study indicated that *B. licheniformis* N1 from the family 18 chitinase exhibited the specific inhibition of fungal spore germination (Lee et al., 2009). Extracellular chitinase production in certain Gram negative bacterial strains, such as a *Pseudomonas* sp. GRC3 and *Chromobacterium* sp. C61 have been shown to play an important role in the *in vitro* growth inhibition of *R. solani* (Arora et al., 2007; Park et al., 2005). In this study, the relationship between chitinolytic ability and biocontrol activity was compared between the wild-type and its mutants. The results suggested that the biocontrol activity of strain C-3 might not be dependant on the ability to produce of chitinolytic enzymes.

Taken together, the newly identified genes of *L. enzymogenes* C-3 might be involved as important components and play different role in the secretion of extracellular

chitinase, and the expression of antifungal traits. This study may provide new information for the in-depth investigation of the regulatory network or components in chitinase production of *L. enzymogenes* C-3 in the future.

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