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Jeffrey D. Palumbo
State University of New Jersey

Gary Y. Yuen
University of Nebraska-Lincoln, gyuen1@unl.edu

C. Christine Jochum
University of Nebraska-Lincoln, charlene.jochum@unl.edu

Kristin Tatum
The State University of New Jersey

Donald Y. Kobayashi
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Mutagenesis of β -1,3-Glucanase Genes in *Lysobacter enzymogenes* Strain C3 Results in Reduced Biological Control Activity Toward *Bipolaris* Leaf Spot of Tall Fescue and *Pythium* Damping-Off of Sugar Beet

Jeffrey D. Palumbo, Gary Y. Yuen, C. Christine Jochum, Kristin Tatum, and Donald Y. Kobayashi

First, fourth, and fifth authors: Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, New Brunswick 08901; and second and third authors: Department of Plant Pathology, University of Nebraska, Lincoln 68583.

Current address of J. D. Palumbo: U.S. Department of Agriculture–Agricultural Research Service, Plant Mycotoxin Research Unit, Albany, CA 94710.

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ABSTRACT

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Lysobacter enzymogenes produces extracellular lytic enzymes capable of degrading the cell walls of fungi and oomycetes. Many of these enzymes, including β -1,3-glucanases, are thought to contribute to the biological control activity expressed by several strains of the species. *L. enzymogenes* strain C3 produces multiple extracellular β -1,3-glucanases encoded by the *gluA*, *gluB*, and *gluC* genes. Analysis of the genes indicates they are homologous to previously characterized genes in the related strain N4-7, each sharing >95% amino acid sequence identity to

their respective counterparts. The *gluA* and *gluC* gene products encode enzymes belonging to family 16 glycosyl hydrolases, whereas *gluB* encodes an enzyme belonging to family 64. Mutational analysis indicated that the three genes accounted for the total β -1,3-glucanase activity detected in culture. Strain G123, mutated in all three glucanase genes, was reduced in its ability to grow in a minimal medium containing laminarin as a sole carbon source. Although strain G123 was not affected in antimicrobial activity toward *Bipolaris sorokiniana* or *Pythium ultimum* var. *ultimum* using in vitro assays, it was significantly reduced in biological control activity against *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet. These results provide direct supportive evidence for the role of β -1,3-glucanases in biocontrol activity of *L. enzymogenes* strain C3.

Lysobacter enzymogenes is a bacterial species characterized for its propensity to lyse fungi and other microorganisms. It is known to produce an abundance of extracellular enzymes such as chitinases and glucanases that are capable of degrading the major cell wall components of fungi and oomycetes. *L. enzymogenes* also produces other factors, such as antibiotics that are antagonistic to the growth of microbes (6,13,42). *L. enzymogenes* strain C3 has demonstrated biocontrol efficacy toward a number of plant diseases (15,27,46–48), of which extracellular degradative enzymes are predicted to play an important role.

A number of observations provide evidence that extracellular enzymes are involved in biocontrol activity of strain C3. Zhang and Yuen (49,50) demonstrated that purified chitinolytic fractions were active against *Bipolaris sorokiniana* spore germination and inhibited plant host infection by the pathogen. In a separate study (27), the *clp* gene, which encodes a global regulator, was found to control lytic enzyme production as well as a number of other factors in strain C3. Mutation of this regulatory gene resulted in complete loss of antimicrobial activity and significant reduction in biological control of *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet. Despite these supportive studies, direct genetic evidence for the role of cell wall-degrading

enzymes such as chitinases and β -1,3-glucanases in biocontrol activity of strain C3 is lacking.

Recently, three β -1,3-glucanases were isolated and characterized from the biocontrol agent *L. enzymogenes* N4-7 (33). The *gluA* and *gluC* genes encode enzymes with catalytic domains belonging to glycosyl hydrolase family 16. The enzymes differ structurally in that the *gluC* gene product contains the presence of a family IV cellulose-binding domain. In contrast, the *gluB* gene product is related to glycosyl hydrolase family 64. The molecular and biochemical characterization of these enzymes in strain N4-7 provided a solid foundation with which to assess the role of β -1,3-glucanases in biocontrol. However, N4-7 is genetically intractable, and this precluded the ability to further analyze β -1,3-glucanase activity by a mutational approach.

Taxonomically, strain N4-7 is closely related to strain C3 and these strains share a number of phenotypic traits (42). Unlike strain N4-7, strain C3 is genetically tractable and, moreover, has been more extensively studied as a biocontrol agent using a number of pathosystems, ultimately providing a better system by which to assess biocontrol traits. In this study, our goals were to identify and characterize β -1,3-glucanase genes within strain C3 and create sequential knockout mutations of each gene to assess a role for the enzyme activity in biocontrol. We describe the characterization of three β -1,3-glucanase genes in *L. enzymogenes* strain C3. We further demonstrate that a strain containing mutations in all three β -1,3-glucanase genes is reduced in biocontrol of *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet.

Corresponding author: D. Y. Kobayashi
E-mail address: Kobayashi@aesop.rutgers.edu

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains and plasmids used in this study are described in the text or are listed in Table 1. A naturally rifampicin-resistant derivative of *L. enzymogenes* strain C3 was used for all experiments. All *L. enzymogenes* strains were maintained on 10% tryptic soy agar (TSA) (Difco Laboratories, Detroit) and grown at 30°C with shaking in medium 813 or medium 813 containing yeast cell walls (813Y) prepared as described (33). Media were supplemented with rifampicin (100 µg/ml), chloramphenicol (50 µg/ml), or gentamicin (100 µg/ml) when appropriate. *Escherichia coli* cultures were grown in Luria Bertani (LB) (Difco Laboratories) broth or agar medium supplemented with ampicillin (100 µg/ml), tetracycline (25 µg/ml), chloramphenicol (25 µg/ml), or gentamicin (12.5 µg/ml) when appropriate.

Identification and analysis of β -1,3-glucanase genes. Digoxigenin (DIG)-labeled oligonucleotide probes *glu31* and *glu43*, previously derived from amino acid sequences of internal fragments of β -1,3-glucanases purified from strain N4-7 culture filtrate (33), were used as probes to identify homologous β -1,3-glucanase-encoding genes in strain C3. A cosmid genomic library of strain C3 DNA maintained in *E. coli* HB101 (27) was screened by Southern hybridizations using standard procedures (37). Hybridizations were performed at 50°C (*glu31* probe) or 65°C (*glu43* probe) and washed under high stringency conditions according to the manufacturer's recommendations (Genius nonradioactive labeling and detection kit; Roche Diagnostics, Indianapolis, IN). Oligonucleotide-hybridizing fragments were subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA) and sequenced on an ABI 373 DNA automated sequencer (Applied Biosystems, Foster City, CA). Sequences were assembled using Lasergene sequence analysis software (DNASTAR, Madison, WI). Open reading frames (ORFs) encoding β -1,3-glucanases were identified by similarity to known bacterial β -1,3-glucanase genes using the Basic Local Alignment Search Tool (BLAST) (1) internet site. Glycoside hydrolase families were determined using the CAZy(ModO) Coutinho and Henrissat (1999) Carbohydrate-Active Enzymes server online.

Mutagenesis of β -1,3-glucanase genes. Mutations in *gluA*, *gluB*, and *gluC* in strain C3 were constructed using the *sacB* positive selection-suicide vector pJQ200SK (36). The *gluA* mutagenic vector was constructed by cloning a 1.1-kb *SalI* fragment of *gluA* from pCG67 into p18Not (19), resulting in pCG6701. A 0.8-kb

SphI fragment containing the chloramphenicol resistance (*cat*) gene of p34S-Cm (9) was inserted into the unique *SphI* site of pCG6701, resulting in pCG6702. The *NotI* fragment of pCG6702, containing the 1.1-kb *gluA* fragment interrupted at its *SphI* site by the inserted *cat* fragment, was cloned into pJQ200SK, resulting in pCG6703.

The *gluB* mutagenic vector was constructed by cloning a 2-kb *NotI* fragment of *gluB* from pCG865 into p18Not, resulting in pCG8650. A 280-bp *SalI* fragment within *gluB* was excised and replaced with the 0.8-kb *cat*-containing *SalI* fragment of p34S-Cm, resulting in pCG8651. The *NotI* fragment of pCG8651 was cloned into pJQ200SK, resulting in pCG8652.

The *gluC* mutagenic vector was constructed by cloning a 1.3-kb *NotI* fragment of *gluC* from pCG41 into pBluescript SK, resulting in pCG410. An *SphI* fragment containing the promoterless *gfp* gene from pGreenTIR (32) was constructed by cloning the 0.9-kb *HindIII* fragment of pGreenTIR into pUC119 (43) oriented opposite the direction of transcription from the *lac* promoter, followed by excision with *SphI*. This *gfp* fragment was inserted into the unique *SphI* site of *gluC* (in the same orientation) in pCG410, resulting in pCG411. The 2.2-kb *NotI* fragment of pCG411 was cloned into pJQ200SK, resulting in pCG412. Mutagenic constructs pCG6703, pCG8652, and pCG412 were conjugated individually into strain C3 via pRK2013 by triparental mating (10) and transconjugants were selected on LB agar containing rifampicin and gentamicin. DNA hybridization to *gluA*/*gluC*- and *gluB*-specific DIG-labeled probes was used to determine the presence of inserted DNA in transconjugants. Transconjugants containing inserted DNA were plated on LB agar containing 5% (wt/vol) sucrose, and transconjugants that grew on this medium were screened for gentamicin sensitivity and for the presence of the inserted fragment within each β -1,3-glucanase gene. Double- and triple-mutant strains were constructed by stepwise conjugation of each mutagenic construct into strain C3, followed by selection and DNA hybridization analysis of recombinant transconjugants for each β -1,3-glucanase gene.

Native polyacrylamide gel electrophoresis. Strain C3 and β -1,3-glucanase mutant strains were grown for 2 days in medium 813Y. Extracellular proteins were concentrated from 5 ml of culture filtrate by adsorption onto a 1-ml Phenyl Sepharose 6 Fast Flow (high sub) column (Amersham Pharmacia Biotech, Piscataway, NJ), followed by elution with 50% acetonitrile in 20 mM Tris-HCl (pH 6.8). The eluate was vacuum dried and resuspended in 200 µl of electrophoresis buffer (43 mM imidazole, 35 mM

TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Lysobacter enzymogenes</i>		
N4-7	Wild-type comparison strain	26,42
C3	Wild-type parental strain, rif resistance	15,42
G1	<i>gluA</i> mutant	This study
G2	<i>gluB</i> mutant	This study
G3	<i>gluC</i> mutant	This study
G12	<i>gluAgluB</i> mutant	This study
G13	<i>gluAgluC</i> mutant	This study
G23	<i>gluBgluC</i> mutant	This study
G123	<i>gluAgluBgluC</i> mutant	This study
<i>Escherichia coli</i>		
DH5 α	Host strain for general cloning	
HB101	Host strain for genomic library	
Relevant plasmid constructs		
pCG4	Cosmid clone containing <i>gluC</i>	This study
pCG41	4-kb <i>PstI</i> fragment containing <i>gluC</i> from pCG4 cloned in pBluescript SK(+)	This study
pCG412	Mutant construction for <i>gluC</i> cloned in pJQ200SK	This study
pCG6	Cosmid clone containing <i>gluA</i>	This study
pCG67	1.6-kb <i>PstI</i> fragment containing <i>gluA</i> from pCG6	This study
pCG6703	Mutant construction for <i>gluA</i> cloned in pJQ200SK	This study
pCG8	Cosmid clone containing <i>gluB</i>	This study
pCG865	2-kb <i>NotI</i> fragment containing <i>gluB</i> cloned in pBluescript SK(+)	This study
pCG8652	Mutant construct for <i>gluB</i> cloned in pJQ200SK	This study

HEPES, pH 7.4). Samples (5 μ l) were loaded onto 6% acrylamide gels containing 0.4% laminarin and electrophoresed in the same buffer at 10 mA on ice for 4 h. β -1,3-Glucanase activity was detected by incubating gels at 37°C for 30 min and staining with 2,3,5-triphenyltetrazolium chloride (34).

Growth curves. Overnight cultures of *L. enzymogenes* strains grown in 10% tryptic soy broth (TSB) were centrifuged at 7,500 rpm for 10 min, washed, and then resuspended in medium 813 supplemented with 0.2% laminarin to a final concentration of $\approx 10^4$ CFU/ml. Cultures were incubated at 30°C with shaking at 250 rpm. Culture samples were recovered at selected time points and dilution plated onto LB agar to determine CFU/milliliter. Growth rates were calculated by plotting population versus time data and determining time (*t*) points spanning exponential growth. The number of generations (*gen*) produced during exponential growth was calculated using the formula $\log_2(\text{population at } t_2 / \text{population at } t_1)$. Generation times were calculated using the formula $(t_2 - t_1) / \text{gen}$.

In vitro antagonism and biocontrol assays. *L. enzymogenes* strain C3 and mutant strains were evaluated for inhibition of hyphal growth on 10% TSA and potato dextrose agar (PDA). Bacterial strains were spot inoculated onto the periphery of an agar plate and a 0.5-cm-diameter mycelial plug taken from an actively growing culture of *B. sorokiniana* or *Pythium ultimum* var. *ultimum* was transferred to the center. Every agar plate contained strain C3 and a no-bacteria control, and each strain was tested on three replicate plates per medium. Cultures with *P. ultimum* and *B. sorokiniana* were incubated at 25°C for 2 and 5 days, respectively, when the diameter of hyphal growth inhibition zones around bacterial colonies were measured.

Wild-type strain C3 and strain G123, mutated in all three glucanase genes, were compared for biocontrol efficacy in two pathosystems, leaf spot of tall fescue, caused by *B. sorokiniana*, and damping-off of sugar beet, caused by *P. ultimum*. Strains with mutations in one or two glucanase genes were not evaluated because they retained glucanase activity. The assay for biocontrol of Bipolaris leaf spot was conducted as described (27,48). Bacterial suspensions (10^8 CFU/ml) were sprayed onto foliage in pots of tall fescue turf to provide approximately 6.5 \log_{10} CFU/g of leaf. Pots sprayed with sterile distilled water served as controls. Treated pots were inoculated 1 day later with conidia of *B. sorokiniana* (5 ml, 5×10^5 conidia/ml), placed in a moisture chamber for 24 h, and then moved to a growth chamber for an additional 4 days. At that time, disease severity (percent leaf area exhibiting lesions) was visually assessed on 10 leaves (each approximately 10 cm in length) collected at random from each pot. Data from the 10 leaves from a pot were averaged to obtain a value for that pot. The experiment was conducted twice, with three replications in one experiment and four in the other. Post-application population levels of strains C3 and G123 on treated leaves were monitored during these experiments using previously reported methods (48). Analysis of variance was performed on all data, and Fisher's least significant difference (LSD) test was used for means separation ($\alpha = 0.05$). Disease severity data were analyzed with and without arcsine transformation; however, because the two methods yielded similar results, only the analyses based on non-transformed data are reported.

Biocontrol efficacy against *Pythium* damping-off was assessed on the basis of sugar beet seed germination and seedling survival in the presence of the pathogen. The bacterial strains were coated onto sugar beet seed (10^7 CFU/seed) as cell suspensions in 1% methylcellulose. Seed coated with 1% methylcellulose alone served as the control. Twenty coated seed were planted into each 150-ml pot containing soil infested with *P. ultimum* var. *ultimum* at approximately 100 propagules/g and premoistened to field capacity. There were three replicate pots per treatment. The percentage of seedlings emerged and surviving were determined daily from the fourth through eighth day after planting. The ex-

periment was conducted twice. Percent emergence or survival data from each experiment were subjected to mixed model analysis of repeated measures data using PROC MIXED (SAS System for Windows, release 8.00; SAS Institute, Cary, NC), with and without arcsine transformation. Fisher's LSD test was used for means separation at $\alpha = 0.05$.

In a separate experiment, strains C3 and G123 were compared for colonization of sugar beet seed and seedling roots. Bacteria-treated seeds were planted into pots of pathogen-free soil that were maintained under the conditions described. Five seed, roots, or both from five seedlings were sampled from each of three pots per treatment at 2-day intervals following planting. Seed were washed in phosphate buffer whereas roots were weighed and then ground in buffer, and then each wash was dilution plated on TSA with rifampicin. Bacterial population levels were expressed as \log_{10} CFU per seed or \log_{10} CFU per gram root in the statistical analysis.

Nucleotide sequence accession numbers. Nucleotide sequences were deposited as GenBank accession nos. AY667477 (*gluA*), AY667478 (*gluB*), and AY667479 (*gluC*).

RESULTS

Identification of β -1,3-glucanase genes in strain C3. Analysis of β -1,3-glucanase activities in culture filtrates of strain C3 on nondenaturing polyacrylamide gels showed that the β -1,3-glucanase activities produced by strain C3 resolved in native gels in a nearly identical pattern to those produced by strain N4-7 (Fig. 1). Both strains produced two β -1,3-glucanase activities that appeared on cathodic gels (Fig. 1A), which correlate to expression of two genes, *gluA* and *gluB*, in strain N4-7 (33). In addition, both strains produced two glucanase activities that appeared on anodic gels (Fig. 1B), which correlate to expression of multiple products from a third gene, *gluC*, in strain N4-7 (33). The oligonucleotide probes *glu31* and *glu43*, which originally were derived from N4-7 glucanase sequences (33), hybridized with high specificity to strain C3 DNA (data not shown), further indicating the similarity of β -1,3-glucanases between the two strains. Using these probes, three sets of clones containing ORFs with high similarity to the *gluA*, *gluB*, and *gluC* genes in strain N4-7 were identified from a cosmid library of strain C3 total DNA.

Probe *glu31* hybridized to a 1.6-kb *Pst*I fragment identified in cosmid clone pCG6 as well as a 4.0-kb *Pst*I fragment identified in cosmid clone pCG4. The 1.6-kb *Pst*I fragment from pCG6 was subcloned in pCG67 and sequence analysis identified a 765-kb ORF designated *gluA*. The 254-residue deduced amino acid sequence encoded by *gluA* is 95% identical to GluA from strain N4-7.

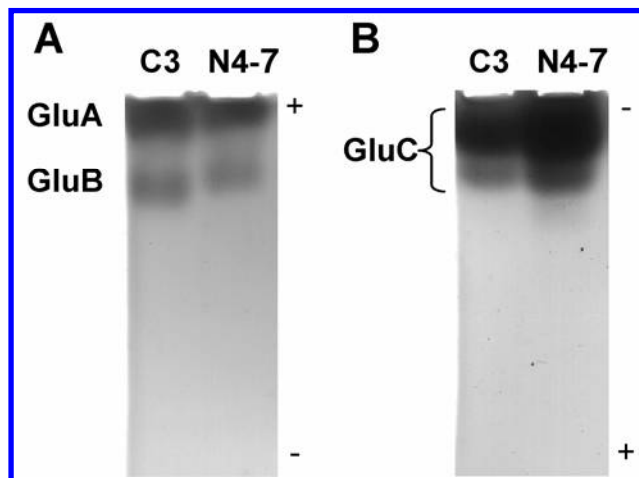


Fig. 1. A, Native cathodic and B, anodic gels depicting β -1,3-glucanase activity from culture filtrates of *Lysobacter enzymogenes* strains N4-7 and C3.

The gene product is structurally organized as a single catalytic domain and includes an N-terminal signal peptide sequence 22 residues long (Fig. 2). The predicted protein contains the motif EIDIME, beginning at residue 150, which includes the catalytic glutamate residues conserved within family 16 glycoside hydrolases (18,20,23,35). The molecular mass of the predicted mature gene product is 26.8 kDa, with a pI of 6.71. A 28-bp G+C-rich sequence including a 12-bp inverted repeat indicative of a transcriptional termination signal was identified beginning 18 bp downstream of the *gluA* ORF.

The 4.0-kb *Pst*I fragment from pCG4 was subcloned into pBluescript SK(+) to give plasmid pCG41. Sequence analysis of this fragment identified a 1,152-kb ORF, designated *gluC*. The 384-residue deduced amino acid sequence encoded by *gluC* is 96% identical to the predicted GluC sequence in strain N4-7 (95% identical at the DNA level). GluC is 83% identical through the first 254 residues to GluA; it contains the conserved EIDIME motif indicative of family 16 glycoside hydrolases and contains a signal peptide sequence 22 residues in length. In contrast to GluA, GluC contains a C-terminal domain with sequence similarity to type IV cellulose-binding domains (CBD IV), as defined by the Simple Modular Architecture Research Tool (SMART) web tool (28,39,40), alternately identified in carbohydrate-binding module family 6 (CBM6) in the Pfam protein families database (41) (Fig. 2). The molecular mass of the mature GluC gene product is predicted to be 40.2 kDa, with a pI of 5.01. Beginning 7 bases downstream of the ORF is a 49-bp sequence of high G+C% predicted to fold in an imperfect stem-loop structure.

Probe *glu43* hybridized to a 2.0-kb *Nor*I fragment identified in cosmid clone pCG8. This fragment was subcloned into pBluescript SK(+) to give plasmid pCG865. Sequence analysis of this fragment identified a 1,188-bp ORF, designated *gluB*. The 395-residue deduced amino acid sequence encoded by *gluB* is 97% identical to the predicted GluB sequence in strain N4-7 (96% identical at DNA level) and includes a 26-residue N-terminal signal peptide. The predicted GluB protein has significant similarity

(30 to 38% identity) to family 64 glycoside hydrolases, for which a conserved catalytic motif has not been defined, and does not contain any detectable binding domains (Fig. 2). The molecular mass of the predicted mature gene product is 39.7 kDa, with a pI of 8.42.

Mutagenesis of β -1,3-glucanase genes. Mutations were constructed in regions encoding the catalytic domains within each of the glucanase genes (Fig. 2), and introduced into the genome of *L. enzymogenes* strain C3 individually and in combination. Single mutant strains G1, G2, and G3, containing mutations in *gluA*, *gluB*, and *gluC*, respectively, failed to produce each respective β -1,3-glucanase as determined by assaying for activity in culture filtrates on nondenaturing activity gels. Similarly, as expected, double-mutant strains G12, G13, and G23 produced only GluC, GluB, and GluA, respectively, whereas mutant strain G123 containing mutations in all three glucanase genes did not produce any of the three β -1,3-glucanase activities (Fig. 3).

To determine the effect of the β -1,3-glucanase gene mutations on the ability of strains to use β -1,3-glucans as a carbon source for growth, all strains were inoculated at $\approx 10^4$ CFU/ml into minimal salts medium supplemented with 0.2% laminarin and generation times were calculated from growth curves of each strain. All tested strains reached $\approx 10^7$ CFU/ml after 24 h (data not shown), at which point differences in growth rate between parent and mutant strains became more pronounced. The generation times of single-mutant strains G1, G2, and G3, and double-mutant strains G12, G13, and G23 were longer than that of strain C3, and maximum populations of the mutant strains were reached 24 to 48 h later than that of strain C3 (Table 2). Maximum population levels for these strains all reached $\approx 10^9$ CFU/ml (data not shown), indicating that expression of at least one β -1,3-glucanase gene would allow *L. enzymogenes* to utilize laminarin as a sole carbon source. In contrast, strain G123, containing mutations in all three β -1,3-glucanase genes, did not grow in this medium beyond initial growth to $\approx 10^7$ CFU/ml (Fig. 4). As a result, the generation time of strain G123 could not be determined.

In vitro antagonism and biocontrol activity of β -1,3-glucanase mutant strains. A mutation of any one or combination of β -1,3-glucanase genes in strain C3 had no effect on inhibition of *B. sorokiniana* or *P. ultimum* hyphal growth in vitro (data not shown). No difference between any mutant and the wild type was found with respect to the diameters of hyphal growth inhibition zones on 10% TSA or PDA.

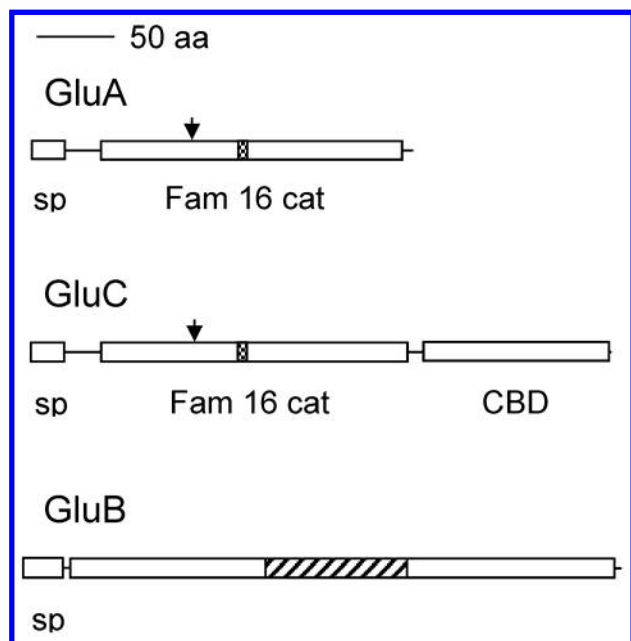


Fig. 2. Structural organization of β -1,3-glucanases from *Lysobacter enzymogenes* strain C3. SP, signal peptide sequence; Fam 16 cat dom, glycosyl hydrolase family 16 catalytic domain; CBD IV, type IV cellulose-binding domain. Hatched regions within GluA and GluC represent the position of the conserved catalytic region containing the amino acid sequence EIDIME. Arrows indicate the insertion sites of *cat* and *gfp* fragments for mutagenesis of *gluA* and *gluC*, respectively. Diagonal lines in GluB represent the region replaced by the *cat* fragment for mutagenesis of *gluB*.

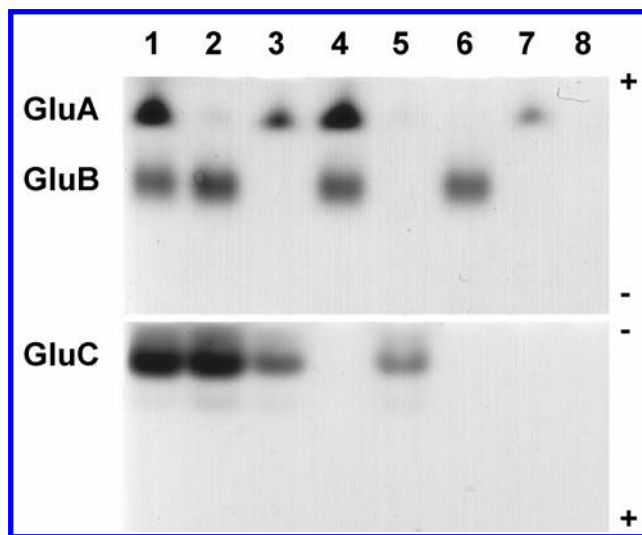


Fig. 3. Native gels depicting β -1,3-glucanase activity from culture filtrates of *Lysobacter enzymogenes* wild-type and mutant strains. Top gel, cathodic; bottom gel, anodic. Lane 1, wild-type strain C3; lane 2, strain G1; lane 3, strain G2; lane 4, strain G3; lane 5, strain G12; lane 6, strain G13; lane 7, strain G23; lane 8, strain G123.

The triple-mutant strain G123 was reduced in biocontrol activity against *B. sorokiniana* compared with wild-type strain C3 (Table 3). In both experiments involving Bipolaris leaf spot on tall fescue, treatment of foliage with strain C3 reduced the severity of leaf spot compared with the water-treated control. The relative effectiveness of strain G123 in suppressing leaf spot, however, varied between experiments. Treatment with strain G123 yielded an intermediate effect in the first experiment, with the disease severity level in the strain G123 treatment being higher than in the strain C3 treatment but lower than in the control treatment. In the second experiment, treatments with strain G123 showed leaf spot severity as high as the control. Strain G123 colonized tall fescue foliage to the same extent as strain C3 (data not shown). No significant difference in population levels of the two strains was found at any sampling date during the experiments. Populations started at $6.5 (\pm 0.3 \text{ standard deviation [SD]}) \log_{10} \text{ CFU/g}$ leaf tissue for C3 and $6.5 (\pm 0.4 \text{ SD}) \log_{10} \text{ CFU/g}$ leaf tissue for G123. At 7 days after inoculation, populations were $6.5 (\pm 0.4 \text{ SD}) \log_{10} \text{ CFU/g}$ leaf tissue and $6.8 (\pm 0.3 \text{ SD}) \log_{10} \text{ CFU/g}$ leaf tissue for strain C3 and G123, respectively.

Biocontrol efficacy of strain G123 against Pythium damping-off of sugar beet also was reduced compared with strain C3 (Fig. 5). In the two experiments conducted, there were highly significant ($P < 0.001$) treatment-day interactions. The wild-type strain C3 improved seedling emergence and survival compared with the control at nearly every reading date. In contrast, treatment with strain G123 had no effect on seedling emergence and survival compared with the control during the earlier phase of both experiments. During the later experiment phase, when numbers of surviving seedlings in the control declined, numbers of seedlings in the G123 treatment increased in comparison, indicating that bacterial factors other than β -1,3-glucanase activity were involved in disease suppression at this stage.

No difference was found between strains G123 and C3 as to their survival on treated sugar beet seed and colonization of roots. From starting populations of $6.9 (\pm 0.2 \text{ SD}) \log_{10} \text{ CFU/seed}$ for strain G123 and $7.0 (\pm 0.2 \text{ SD}) \log_{10} \text{ CFU/seed}$ for strain C3, population levels on treated seed 4 days after planting in soil were $6.5 (\pm 0.4)$ and $6.4 (\pm 0.5 \text{ SD}) \log_{10} \text{ CFU/seed}$, respectively, whereas populations on roots 8 days after planting were $7.2 (\pm 0.7 \text{ SD})$ and $6.9 (\pm 0.4 \text{ SD}) \log_{10} \text{ CFU/g}$ root, respectively.

DISCUSSION

L. enzymogenes strain C3 contains a β -1,3-glucanase system that is nearly identical to that of strain N4-7, as determined by comparisons of enzyme activities and homologous genes. Although the β -1,3-glucanases from strain C3 were not biochemically characterized to the extent of those previously characterized from strain N4-7, the high similarities they share at the genetic level predicts that the enzymes also share similar biochemical features. This includes specificities toward different β -1,3-glucan substrates. GluA and GluC from strain N4-7 have higher relative

specific activity toward linear glucan substrates, whereas GluB had higher specific activity toward the branched chain substrate, zymosan A (33). In the current study, generation times of strains containing mutations in one or two of the three β -1,3-glucanase genes were longer than that of strain C3 in minimal salts medium containing laminarin, a linear β -1,3-glucan, as the sole carbon source (Table 2). In addition, a strain containing mutations in all three β -1,3-glucanase genes was unable to grow on laminarin beyond initial growth observed with all tested strains following transfer from complex medium (Fig. 4). Our interpretation is that the observed initial population increase likely resulted from cellular reserves, whereas the subsequent lack of population increase indicated a lack of ability to utilize laminarin as a substrate; and, thus, the total β -1,3-glucanase activity is encoded by these three genes in strain C3. Together, studies from strains N4-7 and C3 suggest that the production of all three enzymes, each with a different specific activity and substrate specificity, provides for maximal efficiency in degrading complex glucan substrates such as those found in fungal cell walls.

β -1,3-Glucans are critical structural cell wall components in fungi and oomycetes and, in *P. aphaniidermatum*, can comprise >80% of the cell wall polysaccharides (4). Mycolaminarin, a soluble β -1,3-glucan, also serves as the principal cellular storage carbohydrate in the oomycetes (8). Despite the importance and abundance of this group of polysaccharides, β -1,3-glucanases have not been as extensively studied as chitinases and, consequently, their role in biological control has not been as clearly defined. The best evidence that β -1,3-glucanases play important

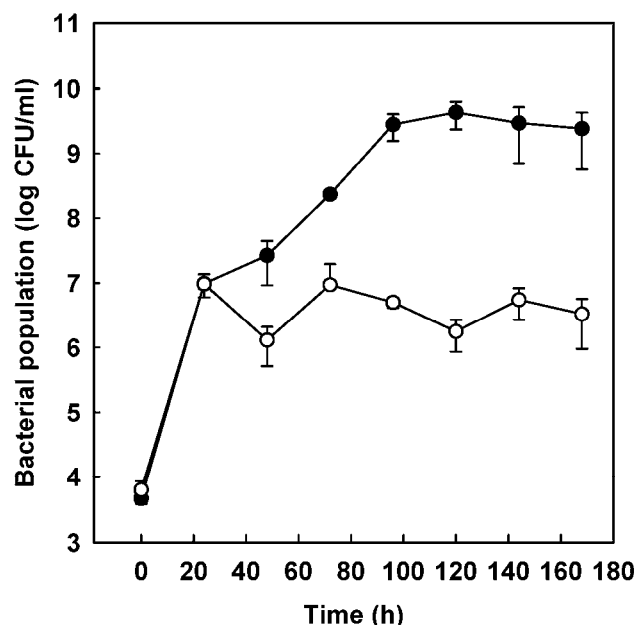


Fig. 4. Growth of *Lysobacter enzymogenes* wild-type strain C3 (closed circles) and β -1,3-glucanase-deficient strain G123 (open circles) on 0.2% laminarin. Error bars represent standard deviation of six replicate samples.

TABLE 2. Growth characteristics of *Lysobacter enzymogenes* wild-type and β -1,3-glucanase mutant strains on 0.2% laminarin^z

Strain	Generation time (h)	Maximum population (log CFU/ml)	Time to reach maximum population (h)
C3	7.22 \pm 1.28	9.60 \pm 0.21	120
G1	9.53 \pm 0.13	9.60 \pm 0.15	168
G2	9.06 \pm 0.81	9.59 \pm 0.18	168
G3	8.03 \pm 0.83	9.46 \pm 0.08	168
G12	9.19 \pm 0.41	9.37 \pm 0.45	168
G13	9.05 \pm 0.71	9.54 \pm 0.07	168
G23	9.62 \pm 0.73	9.53 \pm 0.19	144
G123	ND	6.98 \pm 0.07	ND

^z Mean \pm standard deviation of six replicate samples per experiment; ND = not determined.

TABLE 3. Effects of wild-type strain C3 and β -1,3-glucanase-deficient mutant strain G123 on the severity of Bipolaris leaf spot on tall fescue

Treatment	Diseased leaf area ^z	
	Experiment 1	Experiment 2
C3	11 c	9 b
G123	27 b	16 a
Water control	69 a	19 a
P	<0.001	0.019

^z There were three and four replications in experiments 1 and 2, respectively. Values in a column followed by the same letter are not significantly different at $\alpha = 0.05$ according to Fisher's least significant difference test.

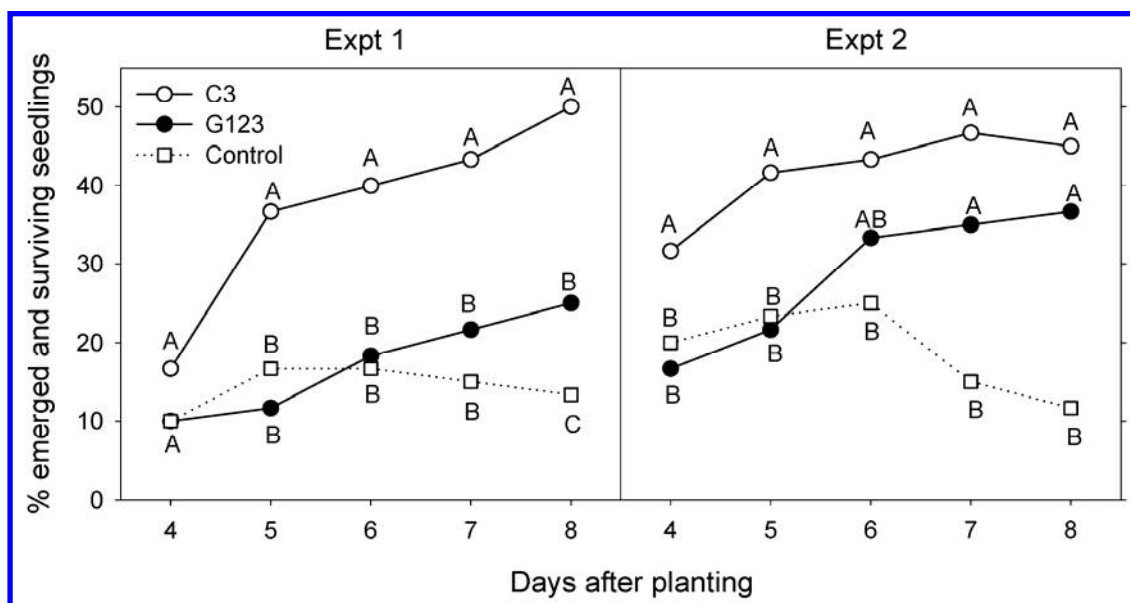


Fig. 5. Effects of seed treatments with wild-type strain C3 and β -1,3-glucanase-deficient mutant G123 on the emergence and survival of sugar beet seedlings in soil infested with *Pythium ultimum* var. *ultimum*. Each data point is a mean of three replications. Values at a given date with the same letter are not significantly different according to the least significant difference test at $\alpha = 0.05$.

roles in biological control were obtained from fungal biocontrol agents such as *Trichoderma* spp. (44), and yeast biocontrol agents (3,5,17). There are far fewer studies correlating β -1,3-glucanases from prokaryotic sources with biocontrol. Fridlender et al. (14) demonstrated fungal lytic activity using concentrated culture filtrates with β -1,3-glucanase activity from a biocontrol strain of *Burkholderia* (*Pseudomonas*) *cepacia*. Other studies have demonstrated similar fungal lytic properties of bacterial glucanases (21,22). Lim et al. (29) implicated a role for glucanase activity in combination with chitinase activity expressed by *Pseudomonas stutzeri* for the control of *Fusarium solani*. However, for each of these studies, only correlative evidence for the role of glucanases in biocontrol was provided. Our results, using a gene-specific mutagenesis approach, provide direct evidence supporting a role for β -1,3-glucanases in biocontrol activity of *L. enzymogenes* strain C3 against *B. sorokiniana* and *Pythium ultimum*. Although only the triple-mutant strain G123 was compared with C3 in our biocontrol experiments, it is likely that subtle differences also exist between strains containing mutations in only one or two β -1,3-glucanase genes. However, these strains were not included in our evaluations due to the variability observed with the biocontrol assays, which would have made any subtle differences between strain treatments difficult to discern.

Several studies have shown exogenous β -1,3-glucanases to lyse hyphae or inhibit hyphal growth in fungi and oomycetes (14, 21,22); therefore, loss of biocontrol activity in the triple-mutant strain G123 most likely was related to reduced antagonism against hyphae. In vitro microbial antagonism, however, was not affected by mutations in the β -1,3-glucanase genes. This discrepancy could have been due to additional antifungal factors expressed by strain C3 masking the effects of β -1,3-glucanases in vitro while having a lesser influence on the phylloplane or in the spermosphere. Alternatively, β -1,3-glucanases could be contributing to biocontrol activity via processes separate from direct antagonism. Oligosaccharide elicitors released by the enzymatic degradation of glucans in fungal and oomycete cell walls can trigger host defenses (7), which is in accord with the induction of resistance in tall fescue to *B. sorokiniana* by strain C3 (25). In addition, the extracellular matrix surrounding conidia and hyphae of *B. sorokiniana*, which is thought to serve adhesive and protective functions, is composed partly of glucans (2); this raises the intriguing possibility that β -1,3-glucanases from strain C3 might

contribute to the biocontrol of *B. sorokiniana* by disrupting the integrity of the extracellular matrix.

Although the lytic nature of *L. enzymogenes* toward other microbes has been described for several years (6,16,24), strains of this bacterial species have been characterized only recently as biocontrol agents for plant diseases (13,42). As a result, many of the mechanisms contributing to microbial antagonism and biocontrol by this bacterial group remain underdetermined. In addition to β -1,3-glucanases, chitinases have been implicated to play an important role in biocontrol activity of *L. enzymogenes* (49,50). Other traits contribute to biocontrol activity as well. A global regulator encoded by the *clp* gene (27) was demonstrated to control several traits in *L. enzymogenes* strain C3, including various extracellular enzymes. Mutations within the *clp* gene resulted in complete loss of both in vitro antimicrobial activity and biocontrol activity. The difference between the *clp* mutation and the directed β -1,3-glucanase mutations in this study as to their impact on biocontrol activity clearly indicates the influence of multiple mechanisms on biological control in this system. Combinations of antifungal traits have been demonstrated to act synergistically to enhance biological control (11,12,30,31,38,45). We predict that mechanisms of strain C3 function similarly, with β -1,3-glucanases acting together with chitinases and other lytic enzymes, as well as antibiotics. Sequential mutation of other proposed biocontrol traits, including chitinase expression, to evaluate combined trait effects are in progress and are expected to provide further insight into roles of these various traits.

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