

A *clp* Gene Homologue Belonging to the Crp Gene Family Globally Regulates Lytic Enzyme Production, Antimicrobial Activity, and Biological Control Activity Expressed by *Lysobacter enzymogenes* Strain C3†

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Lysobacter enzymogenes strain C3, a biological control agent for plant diseases, produces multiple extracellular hydrolytic enzymes and displays antimicrobial activity against various fungal and oomycetous species. However, little is known about the regulation of these enzymes or their roles in antimicrobial activity and biocontrol. A study was undertaken to identify mutants of strain C3 affected in extracellular enzyme production and to evaluate their biocontrol efficacy. A single mini-Tn5-*lacZ*₁-*cat* transposon mutant of *L. enzymogenes* strain C3 that was globally affected in a variety of phenotypes was isolated. In this mutant, 5E4, the activities of several extracellular lytic enzymes, gliding motility, and in vitro antimicrobial activity were reduced. Characterization of 5E4 indicated that the transposon inserted in a *clp* gene homologue belonging to the Crp gene family of regulators. Immediately downstream was a second open reading frame similar to that encoding acetyltransferases belonging to the Gcn5-related *N*-acetyltransferase superfamily, which reverse transcription-PCR confirmed was cotranscribed with *clp*. Chromosomal deletion mutants with mutations in *clp* and between *clp* and the acetyltransferase gene verified the 5E4 mutant phenotype. The *clp* gene was chromosomally inserted in mutant 5E4, resulting in complemented strain P1. All mutant phenotypes were restored in P1, although the gliding motility was observed to be excessive compared with that of the wild-type strain. *clp* mutant strains were significantly affected in biological control of *pythium* damping-off of sugar beet and *bipolaris* leaf spot of tall fescue, which was partially or fully restored in the complemented strain P1. These results indicate that *clp* is a global regulatory gene that controls biocontrol traits expressed by *L. enzymogenes* C3.

The genus *Lysobacter* belongs to the family *Xanthomonadaceae* in the γ -proteobacteria and is characterized by a high G+C content, gliding motility, and a propensity to lyse other microbes, including other bacteria, fungi, and nematodes (5). *Lysobacter enzymogenes* is a species that is characterized by prolific production of extracellular lytic enzymes, including chitinases, β -1,3-glucanases, and proteases. Previously, *Lysobacter* spp. were placed with other bacteria in the myxobacter group. Evidence that extracellular enzymes produced by soil bacteria in this group contribute to lytic activity was reported as early as the 1960s (14, 21). Despite the obvious antagonistic relationships with other microbes, however, few studies have described *Lysobacter* spp. as potential biological control agents for plant diseases.

Three different bacterial strains characterized as biological control agents for plant diseases were recently classified as *L. enzymogenes* (11, 36). Strain 3.1T8 was reported to express antimicrobial activity against fungal and oomycetous plant

pathogens, including various *Pythium* spp. (11). Strain N4-7 was described as a biocontrol agent for summer patch disease of turfgrass caused by *Magnaporthe poae* (22). Strain C3, formerly classified as *Stenotrophomonas maltophilia*, was described as an effective biological control agent for a number of fungal plant diseases, including brown patch of turfgrass caused by *Rhizoctonia solani* (13, 41), leaf spot of tall fescue caused by *Bipolaris sorokiniana* (42), and bean rust caused by *Uromyces appendiculatus* (40). It also displayed in vitro activity against the oomycete *Pythium ultimum* (Yuen, unpublished data). Consistent with the previously described characteristics of *L. enzymogenes*, the three strains express a number of traits thought to contribute to biocontrol activity, including a variety of lytic enzyme activities (11, 12, 36, 43). Indeed, studies with strain C3 have provided strong supporting evidence that activity resulting from multiple isoforms of chitinases has an important functional role in biocontrol (43, 44, 45). Despite the collective evidence, exact roles for various enzyme types in biocontrol activity have been difficult to establish; this has been complicated in part by the existence of different enzymes in multiple isoforms that precludes a mutagenesis approach. In addition, essentially nothing is known about the regulatory mechanisms controlling expression of enzymes and other biocontrol traits in *L. enzymogenes*.

Our primary goals for studying *L. enzymogenes* were to gain a better understanding of the roles of the various extracellular

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enzymes in biological control and to begin to understand the regulatory factors that control expression of these traits. In an effort to achieve these goals, we initiated a transposon mutagenesis project with strain C3 in an attempt to identify mutants that lack extracellular enzyme production. Here we report characterization of a *clp* gene homologue in *L. enzymogenes* strain C3 that belongs to the Crp gene family of global regulators. In plant-associated bacteria, Crp regulators are best known for their involvement in global control of pathogenesis-related traits in *Xanthomonas campestris* pv. *campestris* and *Erwinia chrysanthemi* (9, 31). To date, however, Crp family regulators in biocontrol bacteria have not been well described. In this study, we demonstrated that mutations in the *clp* gene in *L. enzymogenes* C3 affect lytic enzyme production, gliding motility, and in vitro antimicrobial activity. Mutation of the *clp* gene also affects biocontrol activity against pythium damping-off of sugar beet, as well as bipolaris leaf spot of tall fescue.

MATERIALS AND METHODS

Bacterial strains and media. A rifampin-resistant variant of *L. enzymogenes* strain C3 (42) was used for all experiments. C3 and mutant strains were routinely grown at 30°C with shaking in medium 813 (28) containing succinic acid (0.5%, wt/vol) as a carbon source or in Luria-Bertani (LB) broth (Difco) supplemented with 50 µg of chloramphenicol per ml when appropriate. The strains also were cultured on 10% tryptic soy agar (TSA) (Sigma-Aldrich) to produce inocula for biological control experiments. *Escherichia coli* strains HB101 (Gibco-BRL), DH5α (Gibco-BRL), and S17-1(λpir) (34) were grown at 37°C in LB broth supplemented with appropriate antibiotics at the following concentrations unless otherwise noted: chloramphenicol, 25 µg/ml; ampicillin, 100 µg/ml; and tetracycline, 25 µg/ml.

Transposon mutagenesis and screening for loss of extracellular enzyme activities. The transposon mini-Tn5-*lacZ*₁-*cat* was constructed by replacing the kanamycin resistance (Km^r) gene from mini-Tn5-*lacZ*₁ with the *cat* gene encoding chloramphenicol resistance from mini-Tn5-Cm (10). The *cat* gene was excised as a 3.6-kb HindIII fragment from mini-Tn5-Cm and cloned into the HindIII site of p18Not (18). The *cat* gene was reexcised as a NotI fragment and used to replace the 1.6-kb fragment encoding Km^r in mini-Tn5-*lacZ*₁ to obtain mini-Tn5-*lacZ*₁-*cat*.

Mutants of strain C3 were generated by conjugation of the pUT plasmid containing mini-Tn5-*lacZ*₁-*cat* from *E. coli* S17-1(λpir) into strain C3 by using a procedure similar to the procedure described previously (10). Transposon mutants were selected and maintained on LB agar containing 100 µg of rifampin per ml and 50 µg of chloramphenicol per ml. Mutants were initially screened for the loss of lytic enzyme activity on agar media. Chitinase and protease activities were visualized as clearing zones surrounding colonies grown on medium 813 agar containing 1% (wt/vol) colloidal chitin and on medium 813 agar containing skim milk agar, respectively (22). Tween 80 hydrolysis was visualized as a zone of precipitation surrounding colonies grown on LB agar containing 1% Tween 80 (19). Hydrolysis of 1% carboxymethyl cellulose and 0.5% laminarin (soluble β-1,3-glucans) was detected by the appearance of zones of color differentiation surrounding colonies grown on medium 813 agar after staining with 1% Congo red as described previously (8, 15).

Quantification of extracellular enzyme production. Parental and mutant strains were grown for 36 h in medium 813 supplemented with 1% (wt/vol) autoclaved yeast cell walls prepared as described previously (28). Cells were pelleted by centrifugation, and the culture supernatants were retained for enzyme assays. Chitinase activity was assayed by adding 100 µl of culture supernatant to 25 µl of 0.2 M sodium phosphate (pH 7.0), 100 µl of H₂O, and 25 µl of 4 mM *p*-nitrophenyl-*N,N'*-diacetylchitobiose. The reaction mixtures were incubated for 1 h at 37°C, and the reactions were stopped by addition of 0.75 ml of 0.4 M Na₂CO₃. The release of *p*-nitrophenol was calculated by using the molar extinction coefficient of *p*-nitrophenol at A₄₀₅ (18,000 M⁻¹ cm⁻¹). Protease activity was assayed by adding 10 µl of culture supernatant to 100 µl of 1% (wt/vol) azoalbumin in 0.1 M sodium acetate (pH 5.4). The reaction mixtures were incubated for 1 h at 30°C, and the reactions were stopped by addition of 100 µl of cold 10% trichloroacetic acid. The reaction mixtures were stored at 4°C for 1 h, and precipitated azoalbumin was removed by centrifugation. The absorbance

at 366 nm of each reaction supernatant was measured relative to that of an enzyme-free control reaction mixture, and protease activity was expressed as the increase in A₃₆₆ per minute. β-1,3-Glucanase activity was measured by determining the release of reducing sugars from hydrolysis of laminarin as described previously (28). All enzyme activities were assayed in triplicate, and the results were normalized to cell number.

Molecular characterizations and sequence analyses. Restriction digestion, electrophoresis, ligation, and Southern hybridization were performed by using standard procedures (32). A genomic library of mutant strain 5E4 was constructed in cosmid vector pLAFR3 (35) and packaged into lambda phage with Gigapak Gold extracts (Stratagene) by a procedure described previously (23). After phage infection of *E. coli* HB101, cosmid clones containing the mini-Tn5-*lacZ*₁-*cat* insertion were directly selected by plating cells on LB agar containing chloramphenicol and tetracycline. Three overlapping cosmid clones were recovered by this procedure, and restriction digest analysis combined with Southern hybridization analysis indicated that the mini-Tn5-*lacZ*₁-*cat* element was inserted into a 4-kb SalI fragment. One cosmid clone, pFT311, was selected for further characterization. A 6-kb PstI fragment consisting of a portion of the mini-Tn5-*lacZ*₁-*cat* insertion and approximately 3 kb of flanking DNA originating from C3 was subcloned from cosmid pLEC1311 into pUC119, resulting in pLEC4325. This fragment was used as a DNA probe specific for the mutated locus in strain 5E4 by random primer labeling with digoxigenin (Genius nonradioactive labeling and detection kit; Roche Diagnostics). The probe was used in colony hybridization experiments to identify corresponding clones in a cosmid genomic library of strain C3 that were constructed in pLAFR3 as described above for strain 5E4. Four overlapping clones were identified by colony hybridization. A 4-kb SalI fragment from cosmid clone pCAP130 that hybridized to the 5E4 probe was subcloned into pBluescript SK(+) (Stratagene) in both orientations, resulting in plasmids pCAP53 and pCAP55. The nucleotide sequences of both strands of the 4-kb SalI fragment were determined by fluorescence labeling by using a Big Dye version 2 labeling kit with an ABI 3100 DNA automated sequencer (Applied Biosystems Inc.). The sequence of a region consisting of approximately 50 bp and having an unusually high G+C content was not resolved despite repeated attempts with various methods to resolve secondary structure compression.

Sequences were assembled and analyzed by using the DNASTAR sequence analysis software (Lasergene). Database searches with identified open reading frames (ORFs) were conducted by using BLAST (Basic Local Alignment Search Tool) (3) at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>).

Reverse transcription (RT)-PCR of *clp* and acetyltransferase transcript. Two primer sets, shown in Table 1, were designed to evaluate transcriptional expression of the *clp* and acetyltransferase (AT) genes. The first primer set, primers CAF1 and CAR1, was designed to include 45 bases upstream of the start codon of *clp* and 136 bases of the coding region of the acetyltransferase gene and amplified an 878-bp fragment. This primer set spanned the region that the transposon inserted into 5E4. The second primer set, primers CAF2 and CAR2, was designed to amplify a 616-bp product downstream of the transposon insertion in 5E4, which was located 385 bases downstream from the start codon of the *clp* gene and 184 bases downstream from the start codon of the acetyltransferase gene.

Total RNA was isolated by using TRI REAGENT (Sigma) according to the manufacturer's instructions and was subsequently treated with RQ1 RNase-free DNase (Promega). Fifty nanograms of RNA was used as a template for RT-PCRs, which were performed by using a SuperScript one-step RT-PCR kit (Invitrogen). The initial cDNA synthesis was conducted at 48°C for 50 min and was followed by a predenaturation step of 96°C for 3 min, a 95°C melting step for 40 s, a 57°C annealing step for 40 s, and extension at 72°C for 2.75 min, which was repeated for 35 cycles before final extension for 15 min. Control experiments that were used to test for DNA in the RNA isolation procedure were performed simultaneously by substituting *Taq* polymerase for the SuperScript reverse transcriptase-Platinum *Taq* mixture. A positive control for all RT-PCRs was included for all RNA samples by using primers designed for an *scfV* gene homologue known to be expressed independent of *clp* (30).

Construction of deletion mutants and P1, a *clp*-complemented strain of mutant 5E4. The gene replacement system with vector pJQ200sk (29) was used for construction of all gene replacement mutants. The primers used for all constructs are listed in Table 1. Briefly, ca. 500-bp flanking regions spanning the regions of targeted deletions were amplified by PCR by using primers that incorporated unique restriction sites at the ends. Amplified products were cloned into pGEMTeasy (Promega), recovered by digestion with the appropriate restriction enzymes, and cloned into pJQ200sk. The resulting constructs were mated into strain C3 by using *E. coli* S17-1. Colonies that grew on LB agar supplemented with rifampin, kanamycin, and gentamicin (100 µg/ml) represented single-event

TABLE 1. Oligonucleotide primers used for RT-PCR and construction of various mutant strains

Primer	Sequence (5'-3') ^a	Use
CAF1	CGTATTGCCCTAAGCTCGGTTGAA	RT-PCR
CAR1	GCGATGCGCTCGATGGCTTT	RT-PCR
CAF2	GCGCCCAAGCTGCTGTATGC	RT-PCR
CAR2	CGATCTCGGCCAGCAAAAGGTAG	RT-PCR
clpF1	<u>GAGCTCGATCTCCGCCACATCCGTCA</u>	Upstream flank for construction of mutant strains DC211 and DCA2422; SstI
clpR1	<u>GAATTCGAGCCGGAGACGACATAATAGAGG</u>	Upstream flank for construction of mutant strains DC211 and DCA2422; EcoRI
clpF2	<u>GAATTCGCGCCGCTGCTCAAGAAG</u>	Downstream flank for construction of mutant strain DC211; EcoRI
clpR2	<u>CTCGAGGCCAGCGTCTTGACGAACTTG</u>	Downstream flank for construction of mutant strain DC211; SstI
clpF3	<u>GAATTCGCCATACCCGCTACTCGCTGAC</u>	Upstream flank for construction of mutant strain DC2422; EcoRI
clpR3	<u>CTCGAGCGCGGATCCGACATAAG</u>	Downstream flank for construction of mutant strain DC2422; SstI
PIF1	<u>GACGGGCGAGGGCCGCGA</u>	Amplification of <i>clp</i> for construction of P1, complementation strain of mutant 5E4
PIR1	GCGCGTCCCATCGGGGC	Amplification of <i>clp</i> for construction of P1, complementation strain of mutant 5E4

^a The underlined sequences are added restriction enzyme sites for the enzymes indicated in the column on the right.

recombinant strains that contained vector constructs incorporated into the chromosome. Individual colonies were selected and grown overnight at 30°C with shaking in LB broth and then diluted 1:1,000 into fresh LB broth supplemented with 5% sucrose. After 6 h of incubation, cultures were plated onto LB agar supplemented with 5% sucrose and incubated at 30°C. Single colonies were selected and restreaked to obtain single colonies several times before replication onto LB agar containing gentamicin to confirm gentamicin sensitivity and loss of the pJQ200sk vector. Double recombination events in putative mutants were verified by Southern blot hybridization. For construction of a deletion mutation in the *clp* gene, the clpF1-clpR1 and clpF2-clpR2 primer pairs were designed to delete a 445-bp region beginning 182 bases from the predicted ATG start and 60 bases from the stop codon. For construction of a deletion mutation that included a portion of the *clp* gene and the AT gene, the clpF1-clpR1 primer pair was used for one flank and a second primer pair, primers clpF3 and clpR3, was designed to delete a 725-bp region between base 182 of the *clp* gene and base 211 of the AT gene (Table 1; see Fig. 3A).

Due to the lack of a well-developed plasmid vector system for transcomplementation in *L. enzymogenes* strain C3, a *clp*-complementing strain was constructed by chromosomal insertion of the gene into the *sciV* gene. The *sciV* gene is part of a type III secretion system in *L. enzymogenes* strain C3, which to our knowledge is not associated with the *clp* gene or traits regulated by the gene (30). The complementing strain P1 was constructed by using a procedure similar to the procedure used for mutant construction. The *clp* gene was amplified by PCR by using primers PFI, beginning 200 bases upstream of the start codon of *clp*, and PRI, beginning 18 bases downstream of the stop codon of *clp* (Table 1), and was cloned into pGEMTeasy. The *clp* gene was then excised with NotI and cloned into a NotI site located between ca. 500-bp regions flanking the *sciV* gene cloned in pJQ200sk. The resulting construct was transformed into *E. coli* S17-1 and chromosomally introduced into strain C3 as described above.

Gliding motility, glass adherence, and antimicrobial activity assays. Gliding motility was assayed by streaking bacteria onto 10% TSA and incubating the cultures at 30°C as previously described (36). Bacterial adherence to glass was observed by growing cultures of *L. enzymogenes* strains in glass test tubes containing 3 ml of LB at 30°C with shaking for 24 to 72 h.

The in vitro antimicrobial activities of *L. enzymogenes* strains were determined by coculturing bacterial strains with the fungus *B. sorokiniana* isolate Bs1 (42) and the oomycete *P. ultimum* isolate P201 (39). The bacterial strains were spot inoculated on the periphery of 10% TSA plates, and we included a mock-inoculated plate as a control. The center of each plate was inoculated with a mycelial plug containing either *R. solani* or *P. ultimum* (three plates per pathogen). After the plates were incubated for 2 to 5 days at 25°C, the radii of growth inhibition zones around bacterial colonies were measured.

Biological control assays. C3 and mutant strains were compared for biocontrol efficacy in two pathosystems. The biocontrol assay for leaf spot of tall fescue, caused by *B. sorokiniana*, was conducted as described by Zhang and Yuen (42). Bacterial suspensions (10⁸ CFU/ml) were sprayed onto foliage in pots of tall fescue turf 1 day prior to inoculation with conidia of *B. sorokiniana*. Pots sprayed with sterile distilled water served as controls. Inoculated pots (four pots per

treatment) were placed in a moisture chamber for 24 h and then moved to a growth chamber and incubated for an additional 7 days, when the disease severity was visually assessed on the basis of the percentage of the leaf area exhibiting lesions. The experiment was conducted two times. The population levels of strain C3 and 5E4 were monitored during these experiments by collecting leaves from treated turf at various times after bacterial application and plating dilutions of leaf wash on 10% TSA amended with rifampin. The biocontrol assay for pythium damping-off of sugar beet, caused by *P. ultimum*, was similar to the assay described by Osburn et al. (27). The strains were coated onto sugar beet seeds (5 × 10⁸ CFU) as cell suspensions in 1% methylcellulose. Control seeds were coated with 1% methylcellulose alone. Twenty coated seeds were planted into each pot of soil infested with *P. ultimum* at a concentration of approximately 100 propagules/g (16) and premoistened to field capacity. There were five replicate pots per treatment. After the pots were maintained in a growth chamber at 20°C for 5 days, control of *P. ultimum* was quantified by measuring seedling emergence, and the results were expressed as percentages. The experiment was conducted twice.

In both sets of experiments, the results of each experiment were analyzed separately by analysis of variance. Duncan's multiple range test was used for separation of means ($\alpha = 0.05$).

Nucleotide sequence accession numbers. The nucleotide sequence of the 2.8-kb DNA fragment containing the *speD*, *clp*, and acetyltransferase gene homologues from *L. enzymogenes* has been deposited in the GenBank database under accession no. AY316743. The nucleotide sequence of the 1.2-kb fragment containing the *serB* gene homologue and a portion of the *trpC* gene homologue has been deposited under accession no. AY316744.

RESULTS

Mutant strain 5E4 is affected in several phenotypes. More than 2,000 Cm^r isolates recovered from mini-Tn5-*lacZ*₁-*cat* mutagenesis of strain C3 were screened for loss of chitinase, β -1,3-glucanase (laminarinase), protease, carboxymethyl cellulase, and Tween 80 hydrolysis activities by agar plate assays. A single mutant, designated strain 5E4, that was significantly reduced or devoid of all activities in agar plate assays was found (Table 2). Reduction of enzyme functions in 5E4 was confirmed by quantification of chitinase, β -1,3-glucanase, and protease activities relative to the activities of wild-type strain C3 (Fig. 1). Low levels of all three enzyme activities were detected with mutant 5E4 compared with the no-enzyme control; however, expression of all three enzyme activities was reduced in the mutant 10- to 20-fold compared to strain C3.

In addition to enzyme activities, a number of phenotypes,

TABLE 2. Phenotypes of *L. enzymogenes* wild-type strain C3 and mutant strains

Phenotype	Strain				
	C3	5E4	DC211	DCA2422	P1
Enzyme activities ^a					
Chitinase	+	-	-	-	+
β -1,3-Glucanase	+	-	-	-	+
Protease	+	+/-	+/-	+/-	+
Carboxymethyl cellulase	+	+/-	+/-	+/-	+
Tween 80 hydrolysis	+	-	-	-	+
Gliding motility	+	-	-	-	++
Glass adherence	-	+	+	+	-
Antimicrobial activities ^b					
<i>Bipolaris sorokiniana</i>	9 \pm 1	0	0	0	11 \pm 0
<i>Pythium ultimum</i>	8 \pm 0	0	0	0	10 \pm 1

^a In vitro activities observed on agar medium after 1 to 3 days of incubation as described in Materials and Methods. +, zone of activity >2 mm from the colony edge; +/-, zone of activity >0 and <2 mm from the colony edge; -, no detectable zone of activity.

^b Activity expressed as the radius (in millimeters; mean \pm standard deviation) of the hyphal growth inhibition zone surrounding colonies of *L. enzymogenes* on 10% TSA.

including colony morphology on agar media, were altered in mutant 5E4 compared with strain C3. Depending on the medium used for growth, colonies of 5E4 often had a rougher, drier appearance and a deeper yellow pigmentation than C3 (data not shown). Mutant strain 5E4 was also devoid of gliding motility on 10% TSA and lacked the spreading colony phenotype displayed by strain C3 (Fig. 2A and Table 2). In contrast to C3 grown in broth cultures, 5E4 typically formed aggregates and adhered to the glass surface of culture shake tubes and flasks (Fig. 2B and Table 2).

Mutant 5E4 contains a transposon insertion in a *clp* gene homologue belonging to the Crp gene family of regulators. The transposon insertion in 5E4 mapped in a 4-kb *Sa*I fragment, and a sequence analysis of this fragment resulted in identification of four ORFs and a portion of a fifth ORF (Fig. 3A). The transposon insertion in 5E4 mapped between bases 222 and 223 from the start codon of the second ORF, which consisted of 690 bases and was located 1,239 bp from a *Sa*I site. A GenBank database search and pairwise comparisons revealed

that the ORF exhibited more than 75% amino acid identity to the catabolite activator protein-like protein (*clp*) gene from *Xanthomonas campestris* pv. *campestris* (9) and homologues originating from related members of the *Xanthomonadaceae*. Lower levels of similarity were detected with other members of the cyclic AMP receptor protein (*crp*) gene family (40 to 50% identity) from *E. coli* and other distantly related taxa.

Seven bases immediately downstream of the *clp* gene was a second ORF consisting of 462 bases. An RPS-BLAST search of the conserved domain database indicated that the predicted protein product was similar to acetyltransferases belonging to the Gcn5-related *N*-acetyltransferase (GNAT) superfamily (pfam00583; *E* value, 5e-11) (26). Similarity to these acetyltransferases was further supported by the presence of signature motifs associated with members of the GNAT superfamily (Fig. 3B). Surprisingly, in contrast to the *clp* gene homologue, BLAST searches of the acetyltransferase did not reveal similarities to sequences encoded by members of the *Xanthomonadaceae* at either the nucleotide or amino acid level. The *clp* and acetyltransferase ORFs were flanked on both sides by ORFs oriented in the opposite direction (Fig. 3A). The first ORF, located 219 bp upstream of *clp* and consisting of 795 bases, was similar (>88% amino acid identity) to the *speD* gene encoding *S*-adenosylmethionine decarboxylase from *Xanthomonas* and *Xylella* spp. Nothing resembling a true ORF was detected immediately downstream of the putative acetyltransferase gene in a region more than 400 bp long. However, several inverted repetitive sequences indicative of transcriptional termination signals were observed throughout the region.

A short nucleotide sequence region, spanning approximately 50 bp, could not be resolved due to GC compression between the region containing the 400 bp downstream of the putative acetyltransferase and a fourth ORF. This ORF exhibited more than 60% identity at the amino acid level with putative *serB* homologues encoding phosphoserine phosphatase from members of the *Xanthomonadaceae*. Part of a fifth ORF, located 35 bp upstream of the *serB* ORF, was similar (>60% identity) to *trpC* homologues encoding indole-3-glycerol phosphate synthase in members of the *Xanthomonadaceae*, suggesting that

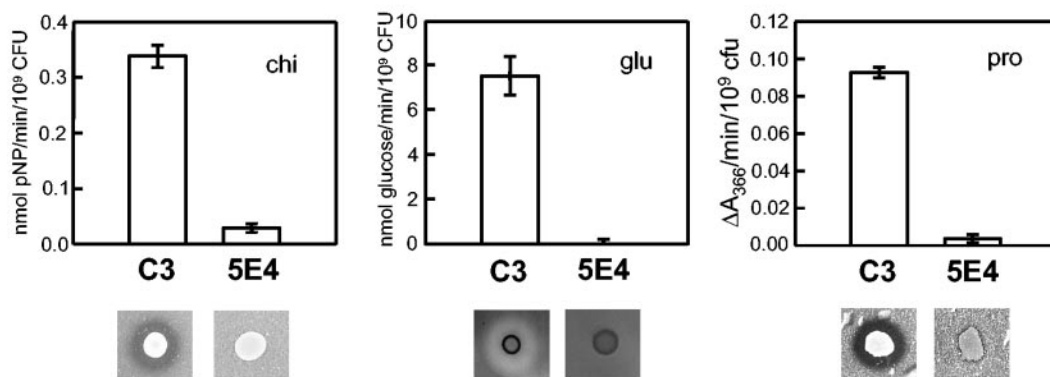


FIG. 1. Specific chitinase (chi), β -1,3-glucanase (glu), and protease (pro) activities in *L. enzymogenes* culture filtrates from strain C3 or 5E4 cultures grown in minimal salts medium 813 supplemented with yeast cell walls. The activity values were standardized to 10^9 CFU, and the data are the means and standard deviations for three replicates. Below the graphs enzyme activity is shown as zones around colonies grown on medium 813 agar supplemented with 2% colloidal chitin (chi), 0.5% laminarin (glu), or 1% skim milk (pro).

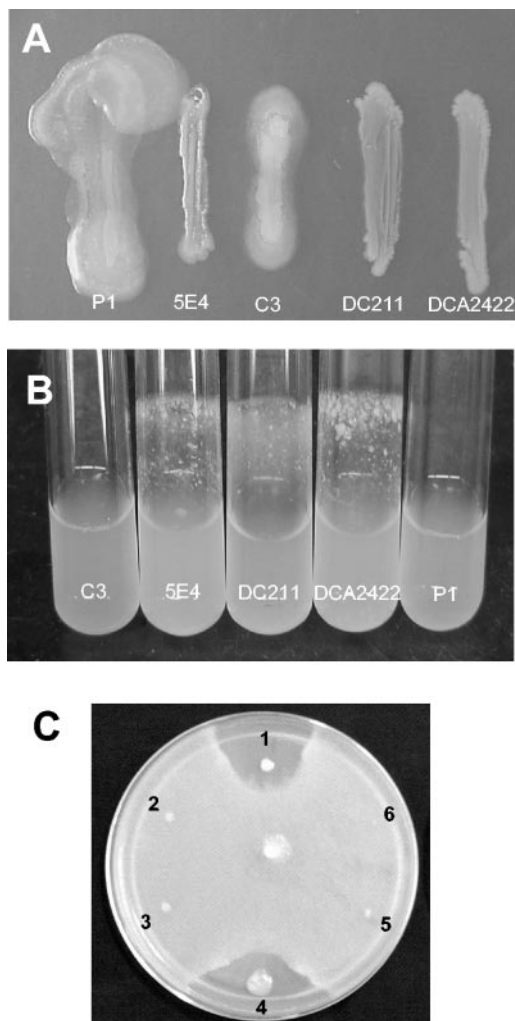


FIG. 2. Phenotypes of *L. enzymogenes* wild-type and mutant strains. (A) Gliding motility assay conducted with 10% TSA. (B) Growth in LB broth, showing the adherence of mutant strains to glass. (C) In vitro antimicrobial activity assay performed with wild-type and mutant strains challenged with *P. ultimum* grown on 10% TSA. Spot 1, C3; spot 2, 5E4; spot 3, DC211; spot 4, P1; spot 5, DCA2422; spot 6, mock inoculation control.

the two ORFs may be part of an operon involved in amino acid metabolism.

***clp* and acetyltransferase ORFs are encoded on a single RNA transcript.** The close proximity of the *clp* and acetyltransferase ORFs to each other suggested that both genes were expressed on a single RNA transcript. To verify this, RT-PCR analyses were conducted by using two different primer sets. The first primer set (primers CAF1 and CAR1) was designed to amplify an 878-bp fragment and to span the entire coding region of *clp* and a portion of the 5' region of the acetyltransferase gene. The second primer set (primers CAF2 and CAR2) was designed to amplify a 616-bp fragment located downstream of the transposon insertion in order to assess the polar effect of the mutation in the *clp* gene in 5E4. This primer set also spanned portions of both the *clp* and acetyltransferase ORFs (Fig. 3A). Amplified RT-PCR products of the expected sizes were obtained with both primer sets when RNA extracted

from C3 was used, indicating that there was a single RNA transcript that spanned the region containing both the *clp* and acetyltransferase ORFs. In contrast, no RT-PCR products were observed when the same primer sets were used with RNA extracted from 5E4 (Fig. 4).

Deletions within the *clp* gene and *clp*-AT gene loci and gene complementation verified the 5E4 mutant phenotype. To verify that the phenotypes observed with mutant 5E4 were due to disruption of the *clp*-AT locus by insertion of mini-Tn5-*lacZ*₁-*cat*, deletion mutations in the *clp* gene and a region spanning the *clp* and AT genes were chromosomally inserted into strain C3. Strains DC211 and DCA2422 contained deletion mutations in the *clp* gene and *clp*-AT genes, respectively (Fig. 3A). These strains were similar to 5E4 for all phenotypes tested (Table 2).

To further verify that the 5E4 mutant phenotype was due to disruption of the *clp* gene, the mutation in 5E4 was complemented by chromosomal insertion of the *clp* gene, creating strain P1. All altered phenotypes of 5E4 were restored in P1, except that it displayed excessive gliding motility compared to wild-type strain C3 (Fig. 2A and Table 2).

Antimicrobial activity and biological control of plant diseases are affected in mutants disrupted in the *clp* gene. Antimicrobial activity against the plant pathogens *B. sorokiniana* and *P. ultimum* was clearly evident from the zones of growth inhibition observed around colonies of strains C3 and P1 on 10% TSA. In contrast, growth of *B. sorokiniana* or *P. ultimum* was not inhibited around colonies of mutant strains 5E4, DC211, and DCA2422 (Fig. 2C and Table 2).

In experiments to evaluate the effects of strains on bipolaris leaf spot, treatment of tall fescue leaves with parent strain C3 or with P1 reduced the leaf spot severity by more than 50% compared with the control. In contrast, the disease severity on leaves treated with mutant strain 5E4, DC211, or DC2422 did not differ significantly from the disease severity in the control plants (Fig. 5A). Throughout these experiments, the sizes of the populations of C3 and 5E4 on leaves ranged from around 7.0 to 8.4 log CFU/g; there was no significant difference between the leaf populations of the two strains on any sampling date (data not shown).

Seed treatment with C3 improved the emergence of sugar beet seedlings in soil infested with *P. ultimum*, whereas treatment with mutants 5E4, DC211, and DCA2422 resulted in no significant difference compared with the methylcellulose-treated control in two separate experiments (Fig. 5B). In contrast, treatment with strain P1 resulted in an intermediate response, in which the seedling emergence with P1 treatment was significantly greater than the seedling emergence with *clp*-disrupted mutant strains but less than the seedling emergence with wild-type strain C3.

DISCUSSION

Members of the *Lysobacter* group characteristically express several traits thought to contribute to antagonism of fungi and other microbes. These traits include production of extracellular degradative enzymes, such as chitinases, glucanases, and proteases, as well as production of antibiotic compounds (5, 20, 36, 37). Mutations in the *clp* gene had a global effect on a variety of *L. enzymogenes* phenotypes, including significant re-

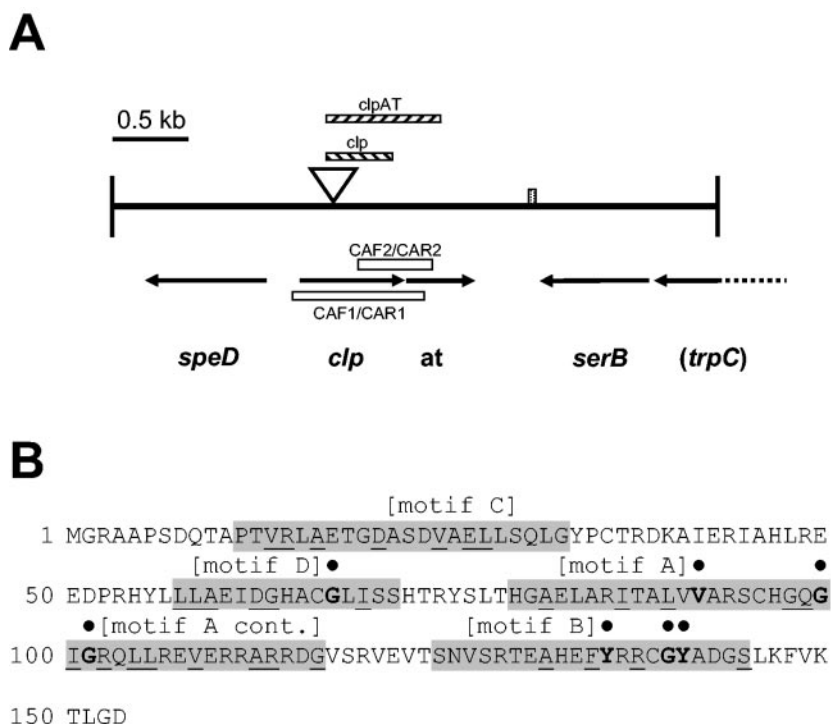


FIG. 3. (A) Map of the 4-kb *SalI* fragment containing mini-Tn5-*lacZ*₁-*cat* in mutant 5E4. The speckled box indicates a region where the sequence is unresolved. The arrows indicate the directions of ORFs; the corresponding gene designations are indicated below the arrows (*at*, acetyltransferase gene). The dashed arrow indicates the partial ORF for *trpC*. The open triangle indicates the position of the transposon insertion in 5E4. The striped boxes above the open triangle indicate deleted regions in the *clp* and *clp-AT* loci in DC211 and DCA2422, respectively. The open boxes indicate the positions of RT-PCR regions when the CAF1-CAR1 and CAF2-CAR2 primer sets were used. (B) Deduced amino acid sequence of the acetyltransferase, with the signature motifs (motifs A to D) of GNAT superfamily members highlighted. The residues in boldface type with solid circles above them are highly conserved, and the underlined residues are moderately conserved.

ductions in extracellular enzyme production, antimicrobial activity, and biocontrol activity. These observations provide further support for the hypothesis that extracellular factors, including lytic enzymes and possibly secondary metabolites such as antibiotics, play important roles in the biocontrol activity of *L. enzymogenes* strain C3. Chitinases have been implicated previously in the biological control activity displayed by *L. enzymogenes*. Zhang and Yuen (44) demonstrated that chitinases purified from culture filtrates enhanced biocontrol of bipolaris leaf spot by strain C3. In much earlier studies, Gillespie and Cook (14) described an enzymatic lytic fraction termed lysin from *L. enzymogenes* strain 495 (formerly classified as a member of the myxobacter group) that displayed activity against bacteria and nematodes. Although the nature of this fraction is unclear, its presence is consistent with antagonistic effects of extracellular factors produced by the species.

Global regulation of traits associated with biocontrol has been best described for fluorescent pseudomonads, such as *Pseudomonas fluorescens*. The regulators involved at the transcriptional level consist of components belonging to complex signal transduction pathways, including two-component systems, alternate sigma factors, RNA binding proteins, and regulatory RNAs (1, 6, 12, 17, 24, 33). Global regulators belonging to the Crp family have not been extensively described in *P. fluorescens* or other plant biocontrol bacteria, but they have been characterized in the plant pathogens *X. campestris* and *E.*

chrysanthemii (9, 31). In both of the latter cases, the regulators controlled numerous traits important in the pathogenic process. The *vfr* gene characterized in *Pseudomonas aeruginosa* is also a member of the Crp gene family and regulates a variety of virulence traits, including production of exotoxin A, pyocyanin, and elastase, production of type IV pili and twitching motility, and quorum sensing (2, 4, 38). While *vfr* gene homologues have been identified in genomic sequences of *P. fluorescens* strains (Kobayashi, unpublished data), no experimental

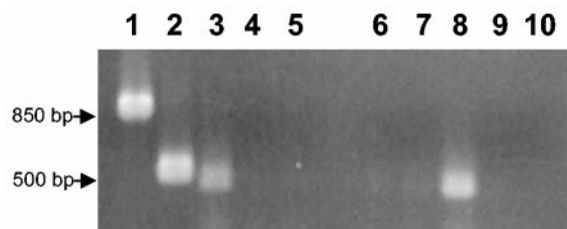


FIG. 4. RT-PCR products from *L. enzymogenes* wild-type strain C3 (lanes 1 to 5) and mutant strain 5E4 (lanes 6 to 10) when constructed primers that span the *clp* and acetyltransferase ORFs were used. The CAF1-CAR1 primer set was used for lanes 1, 4, 6, and 9, and the CAF2-CAR2 primer set was used for lanes 2, 7, 5, and 10. Lanes 4, 5, 9, and 10 contained DNA-negative controls for the primer sets. Lanes 3 and 8 contained RT-PCR-positive controls in which primers for a gene (*scfV*) known to be expressed independent of the *clp* and acetyltransferase genes in *L. enzymogenes* were used.

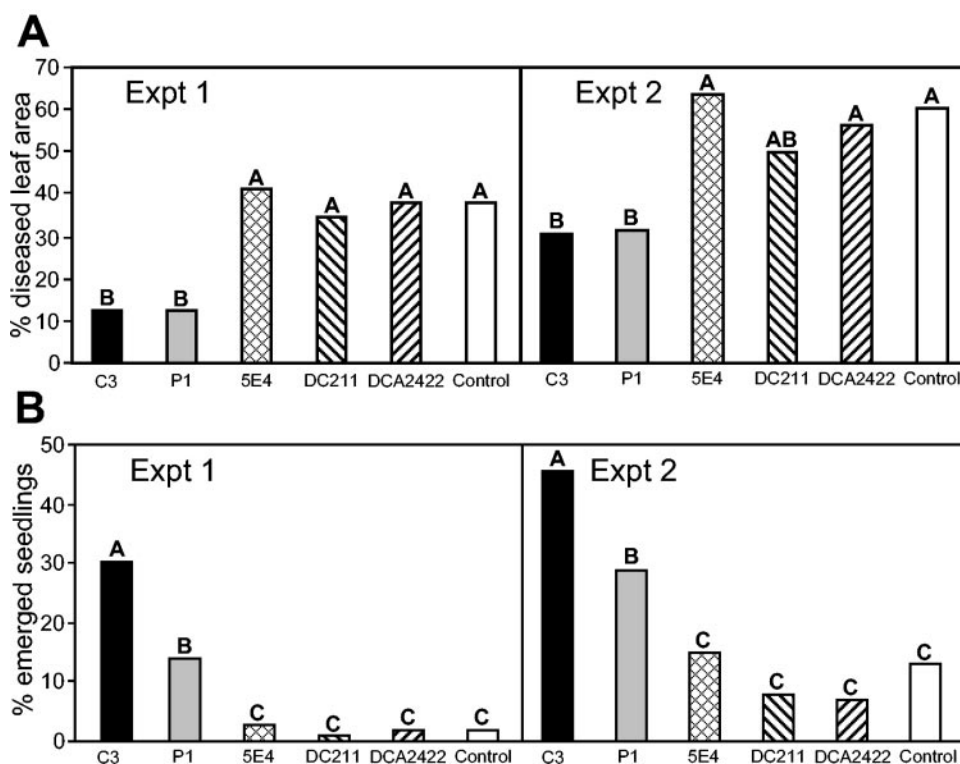


FIG. 5. Biocontrol effects of *L. enzymogenes* strains on the severity of leaf spot caused by *B. sorokiniana* on tall fescue (A) and on pythium damping-off of sugar beet as measured by seedling emergence (B). In each experiment, bars with same letters are not significantly different at $\alpha = 0.05$.

evidence linking the regulator to expression of biocontrol traits has been obtained yet.

The mutation in strain 5E4 affected not only expression of *clp* but also a putative acetyltransferase belonging to the GNAT superfamily. Although a role for the acetyltransferase in gene regulation was not established in this study, it is conceivable that both genes could be involved in a complex regulatory pathway. GNAT acetyltransferases are ubiquitous among organisms and have been associated with a number of cellular functions, including transcriptional regulation (26). To date, however, GNAT superfamily members that function in gene regulation have not been as extensively characterized from prokaryotic sources as they have been from eukaryotic sources.

Several similarities between the *clp* gene previously characterized from *X. campestris* pv. *campestris* and the *clp* gene from *L. enzymogenes* C3 are evident. The physical linkage between the *speD* and *clp* genes in C3 is identical to that described for *X. campestris* pv. *campestris* (25). In addition, the *clp* gene in *X. campestris* pv. *campestris* was found to globally regulate production of various extracellular enzymes, pigmentation, and the extracellular polysaccharide xanthan gum (9). Our observations of altered colony color and morphology in mutant 5E4 are consistent with *clp*-mediated changes in pigmentation and extracellular polysaccharide production. Furthermore, the reduction in fungal antagonism in mutant 5E4 parallels the finding that the phytopathogenicity of the *X. campestris* pv. *campestris* *clp* mutant strain was significantly reduced (9).

Despite the similarities noted above, one distinct difference

is the absence of apparent acetyltransferase homologues in *X. campestris* pv. *campestris* and other related members of the *Xanthomonadaceae*, including *Xanthomonas* and *Xylella* spp. whose genomic sequences have been determined. This finding suggests that the organizational relationship and quite possibly a functional relationship between the *clp* gene and the acetyltransferase gene are unique to *L. enzymogenes*, at least among members of the *Xanthomonadaceae*.

Traits controlled by the *clp* regulator were found to be responsible for biocontrol activity against bipolaris leaf spot of tall fescue and pythium damping-off of sugar beet. The finding that wild-type strain C3 and mutant strain 5E4 were similar in terms of colonization of tall fescue leaves supports the hypothesis that the lack of bipolaris leaf spot biocontrol ability in 5E4 and other *clp* mutants is due to reduced production of extracellular factors rather than impaired colonization ability. For both disease assays, host infection relies on a spore-based inoculum that is activated rapidly and synchronously; disease suppression in these scenarios could be achieved largely or entirely by inhibition of spore germination by extracellular antimicrobial products of strain C3.

The nature of the affected regulatory *clp* gene and the control that it has on various traits has complicated genetic complementation of mutant 5E4. Unlike the *crp* counterparts that have been characterized in enteric bacteria, *clp* functions in *X. campestris* pv. *campestris* independent of cyclic AMP to control gene expression. We have obtained evidence that in *L. enzymogenes* strain C3 expression of chitinases is cyclic AMP dependent, while expression of β -1,3-glucanases is cyclic AMP

independent (Zhou, Reedy, Yuen, and Kobayashi, *Phytopathology* **92**:S91, 2002).

Complementation of strain 5E4 required chromosomal insertion of the *clp* gene, because a stable plasmid system for strain C3 was not present. All phenotypes tested were complemented in the resulting strain, P1, except that in this strain at least two phenotypes were altered compared to wild-type strain C3. P1 displayed excessive gliding motility (Fig. 2A), and biocontrol of pythium damping-off was not fully restored to the levels achieved with strain C3 (Fig. 5B). Based on the chromosomal insertion of *clp* in strain P1, it is possible that these altered phenotypes may have resulted from (i) altered expression of the *clp* gene compared to the wild-type strain, (ii) lack of complementation of the acetyltransferase, or (iii) a second mutational effect that occurred due to the position at which the *clp* gene was placed in P1. The latter explanation is intriguing, since identification in C3 of a type III secretion pathway (30; Reedy and Kobayashi, *Phytopathology* **91**:S75, 2001), a central component for bacterial pathogenicity for eukaryotic hosts (7), suggests that there is a possible pathogenic interaction that also contributes to the biocontrol activity of C3 against *P. ultimum*. Nonetheless, observations made in this study speak to the complexity of the regulatory network governing factors contributing to the antimicrobial activity expressed by *L. enzymogenes*, as well as the factors involved in the interactions between the bacterium and fungal or oomycetous hosts. Efforts are in progress to further define steps in the regulatory pathway and to identify roles of specific traits associated with antimicrobial and biocontrol activities expressed by *L. enzymogenes*.

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