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Both extracellular chitinase and a new cyclic lipopeptide, chromobactomycin, contribute to the biocontrol activity of *Chromobacterium* sp. C61

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SUMMARY

Chromobacterium sp. strain C61 displays antifungal activities *in vitro* and has been used successfully for the biocontrol of plant diseases under field conditions. In this study, the roles of extracellular chitinase and an antifungal compound produced by strain C61 were investigated to elucidate their contributions to biological control activity. The bacterium possessed a locus *chi54* encoding an extracellular chitinase, and mutation of *chi54* eliminated chitinase production. Production of the extracellular enzyme and expression of the *chi54* transcript were increased in the wild-type strain when chitin was added to the culture medium. *In vitro* assays showed that purified chitinase inhibited spore germination of multiple pathogens. However, the *in planta* biocontrol activity of filtrates of cultures grown in the presence of chitin was lower than that of filtrates grown without chitin, indicating that correlation between chitinase and biocontrol activity was lacking. The analysis of C61 culture filtrates revealed an antifungal cyclic lipopeptide, chromobactomycin, whose structure contained a unique nonameric peptide ring. The purified chromobactomycin inhibited the growth of several phytopathogenic fungi *in vitro*, and plant application significantly reduced disease severity for several pathogens. Furthermore, the production of chromobactomycin was reduced in cultures amended with chitin. These data suggest that the production of both the extracellular chitinase Chi54 and the newly identified antibiotic chromobactomycin can contribute, in an interconnected way, to the suppression of plant disease by *Chromobacterium* sp. strain C61.

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INTRODUCTION

Diverse rhizobacteria promote plant growth and/or suppress plant diseases (Lugtenberg and Kamlikova, 2009). The mechanisms involved in plant disease suppression are mediated by a variety of secreted products thought to contribute to antibiosis, competition and the induction of host resistance. The large majority of studies of individual biocontrol agents highlight only a single mode of action, despite the recognition that the most successful agents probably work at the field scale through multiple mechanisms (Kim YC *et al*., 2011). The contributions of different mechanisms, expressed by a single agent, to the emergent property of biological control remain relatively understudied.

Lytic enzymes that degrade fungal cell walls, particularly extracellular chitinases, contribute to pathogen suppression (Dahiya *et al*., 2006; Kobayashi *et al*., 2002). Chitinase-producing bacterial strains are studied because the enzyme (EC 3.2.1.14) hydrolyses the β-1,4-linkage of chitin, a polymer widely distributed in phytopathogenic fungal cell walls (Flach *et al*., 1992). Chitinases are grouped into families depending on the similarities of the amino acids in their catalytic domain (Cohen-Kupiec and Chet, 1998). Plant chitinases and chitinases from *Streptomyces* sp. belong to family 19, which function as endochitinases, have lysozyme activity and are antifungal (Watanabe *et al*., 1999). Most bacterial chitinases are classified into family 18 and show less antifungal activity than that of family 19 chitinases (Dahiya *et al*., 2006). However, studies indicate that some biocontrol-active rhizobacteria produce family 18 chitinases that inhibit the growth of plant pathogenic fungi (Arora *et al*., 2007; Kobayashi *et al*., 2002; Li *et al*., 2008).

Antibiotics and other metabolites produced by different biocontrol bacteria also suppress phytopathogenic fungi, both directly and indirectly. Pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol (DAPG) and phenazines are implicated in

biocontrol mediated by fluorescent pseudomonads (Dubuis *et al*., 2007). Cyclic lipopeptides (CLPs) are produced by many bacterial genera and can show lytic and growth-inhibiting properties against various plant pathogenic pathogens (Raaijmakers *et al*., 2010). For example, CLPs produced by biocontrol *Bacillus* species, including fengycins and iturin, are antifungal against *Fusarium graminearum* (Wang *et al*., 2007), *Colletotrichum dematium* (Hiradate *et al*., 2002) and *Magnaporthe grisea* (Tendulkar *et al*., 2007). However, compounds such as DAPG and CLPs can be multifunctional, affecting both pathogens and plant hosts in a variety of ways, e.g. affecting host defence status and altering rooting patterns (Kim YC *et al*., 2011).

We have identified a root-colonizing *Chromobacterium* sp. C61 as a useful biocontrol agent using chitinase production as a criterion for selecting and testing biocontrol bacteria (Kim *et al*., 2008). Formulations of *Chromobacterium* sp. C61 were used in combination with other culture methods to control *Alternaria* blight and anthracnose of ginseng (Kim *et al*., 2010) and *Phytophthora* blight of pepper (Kim *et al*., 2008) in the field. We presumed that the chitinase activity of C61 accounted for its potent ability to inhibit *Rhizoctonia solani* growth (Park *et al*., 2005). The major extracellular chitinase from the bacterium had a molecular weight of 54 kDa and a pI of 8.7 (Park *et al*., 2007). Our recent draft genome sequence analysis of *Chromobacterium* sp. C61 identified a single chitinase gene (*chi54*) (Kim HJ *et al*., 2011), and site-directed mutagenesis of this gene was performed to identify the key amino acids involved in chitinase activity. For example, replacing the tyrosine at amino acid 218 with a serine in the Chi54 protein enhances chitinase activity (Park *et al*., 2007).

In this study, we examined the role of the major chitinase and a newly identified antifungal compound in the biological control of different plant diseases by *Chromobacterium* sp. strain C61. Our results show that a novel CLP secreted by *Chromobacterium* sp. strain C61, and an extracellular chitinase, contribute in an interconnected way to both *in vitro* antifungal and *in planta* biocontrol activities.

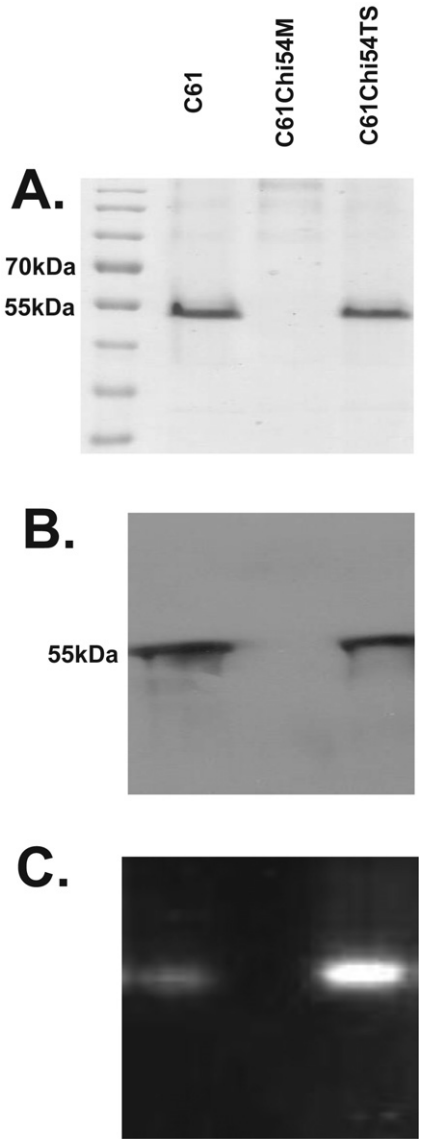

Fig. 1 Comparison of chitinase activity from the *chi54* mutant (C61Chi54M) and the mutant (C6IChi54TS) compared with the wild-type (C61) strain of *Chromobacterium* sp. C61. Coomassie blue staining (A), Western blot (B) and chitinase activity staining (C) of proteins from C61 separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins (20 μg) were partially purified from culture filtrates of the wild-type and mutant strains (C61, C61Chi54M and C61Chi54TS) grown in Luria–Bertani (LB) broth containing 0.2% colloidal chitin.

RESULTS

Biocontrol activities of the *Chromobacterium* sp. C61 chitinase mutants

Strong chitinase production by the wild-type strain was confirmed using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining for activity and Western analysis using a polyclonal antibody that detects the C61 secreted chitinase (Fig. 1). Insertional inactivation of a *chi54* locus in mutant C61Chi54M was verified by polymerase chain reaction (PCR) analysis of the sequence of the mutated gene (data not shown). This mutant did not grow on chitin as a sole carbon source, and Chi54 activity was not detected by activity staining or antibody recognition (Fig. 1). A mutant producing a more active chitinase, C61Chi54TS, as a result of a site-directed mutation that replaced a serine residue with a tyrosine (Park *et al*., 2007), showed greater activity than the wild-type (Fig. 1C).

Culture filtrates of the wild-type *Chromobacterium* sp. C61 and the mutant C61Chi54TS both produced significant *in planta* biocontrol activity against rice blast, tomato leaf blight and wheat leaf rust ($P < 0.05$ for each; Fig. 2). In contrast, the filtrates from

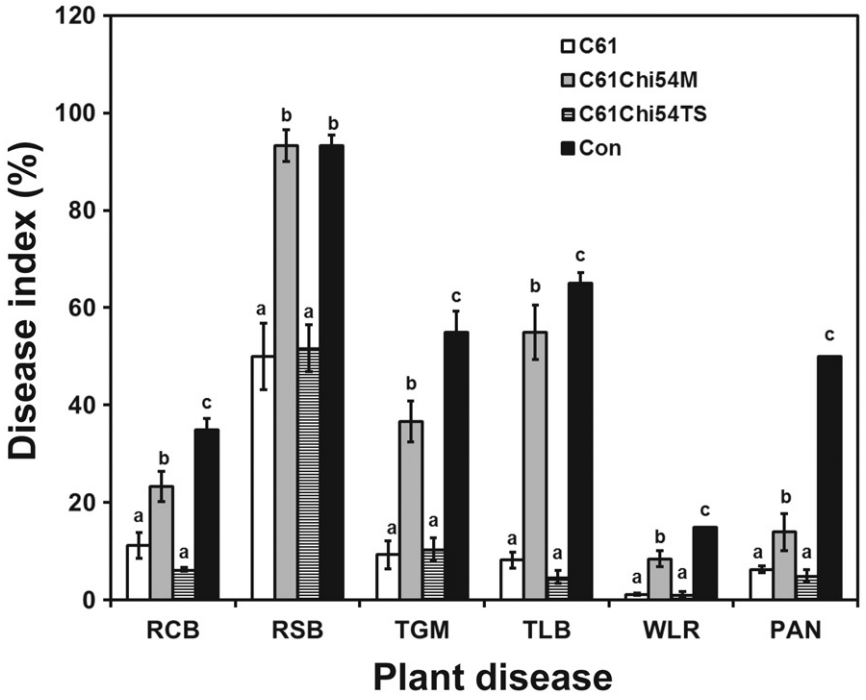

Fig. 2 *In planta* biocontrol efficacies of the extracellular products of *Chromobacterium* sp. C61 and its chitinase mutants. Intact plants were sprayed with cell-free bacterial cultures of the C61 wild-type(C61), C61Chi54M and C61Chi54TS (1:3 dilution), and water was used as the negative control treatment. The disease index resulting from each pathogen was based on the areas of the infected lesion. PAN, red pepper anthracnose; RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mould; TLB, tomato late blight; WLR, wheat leaf rust. Data are means ± standard deviations of three independent experiments with nine plants per treatment. Bars labelled with different letters for each pathosystem have significantly different ($P < 0.05$) levels of disease based on Duncan's multiple range test.

the C61Chi54M mutant showed reduced activity (Fig. 2). Thus, the biological control activity of C61 can be attributed, in part, to the levels of extracellular chitinase activity produced.

Effect of chitin on chitinase expression, and *in vitro* and *in planta* biocontrol of C61

Transcriptional analysis identified *chi54* transcripts in the stationary phase in wild-type cells grown in the presence of colloidal chitin (Fig. 3A). Extracellular chitinase was detected only when the wild-type and C61Chi54TS mutant were grown with chitin and, as expected, there was no detectable extracellular chitinase activity with the C61Chi54M mutant (Fig. 3B). However, *in vitro*, antifungal activity against the fungal plant pathogens *R. solani* and *Botrytis cinerea* by the wild-type *Chromobacterium* sp. C61 was higher on potato dextrose agar (PDA) without chitin (Fig. 3C), indicating that chitinase induction reduced the overall antibiotic-like activity on the plates. In addition, no significant difference was observed between the extent of antagonism between the C61Chi54TS construct and wild-type C61, although the C61Chi54M mutant was less antagonistic.

To test the potential of the chitinase enzyme to inhibit fungal growth, the antifungal activity of purified Chi54 was tested *in vitro*. After incubation for 10 h, spore germination from most tested fungal pathogens was decreased significantly ($P < 0.05$) with 2 and 0.2 mg/mL purified Chi54. No antagonism for *Fusarium oxysporum*, *Colletotrichum gloeosporioides* or *B. cinerea* was observed below 0.2 mg/mL (Fig. S1, see Supporting Information). Germination of *Cladosporium spharospermum* was the most sensitive to Chi54 treatment, and conidial germination of *F. oxysporum* was the most resistant.

Subsequent *in planta* bioassays using cell-free extracts of *Chromobacterium* sp. C61 supported the differential effects seen *in vitro*. For example, foliar application of the cell-free supernatant of C61 grown in potato dextrose broth (PDB) significantly reduced the symptoms of rice blast, rice sheath blight, tomato gray mould, tomato leaf blight and wheat leaf rust (Fig. 4). However, control levels declined when the growth medium was amended with chitin (Fig. 4). These data indicated that components other than Chi54 secreted by C61 contributed to its *in vitro* and *in planta* biocontrol activities.

Identification of the antifungal compound produced by C61

A new antifungal metabolite from wild-type C61 was extracted with ethyl acetate from culture medium. High-performance liquid chromatography (HPLC) analyses of these extracts from cultures of the wild-type indicated that a product (elution time, 8.5 min) had high antifungal activity (Fig. S2, see Supporting Information). Its structure was analysed using two-dimensional ^{1}H–^{1}H correlation spectroscopy (2D ^{1}H–^{1}H COSY), ^{1}H–^{1}H rotating frame nuclear Overhauser effect spectroscopy (^{1}H–^{1}H ROESY), ^{1}H–^{1}H total correlation spectroscopy (^{1}H–^{1}H TOCSY), ^{1}H–^{13}C heteronuclear multiple-quantum correlation spectroscopy (^{1}H–^{13}C HMQC), ^{1}H–^{13}C heteronuclear multiple-bond correlation spectroscopy (^{1}H–^{13}C HMBC), distortionless enhancement by polarization transfer (DEPT) and ^{13}C spectroscopy in methanol-d_4 and dimethyl-

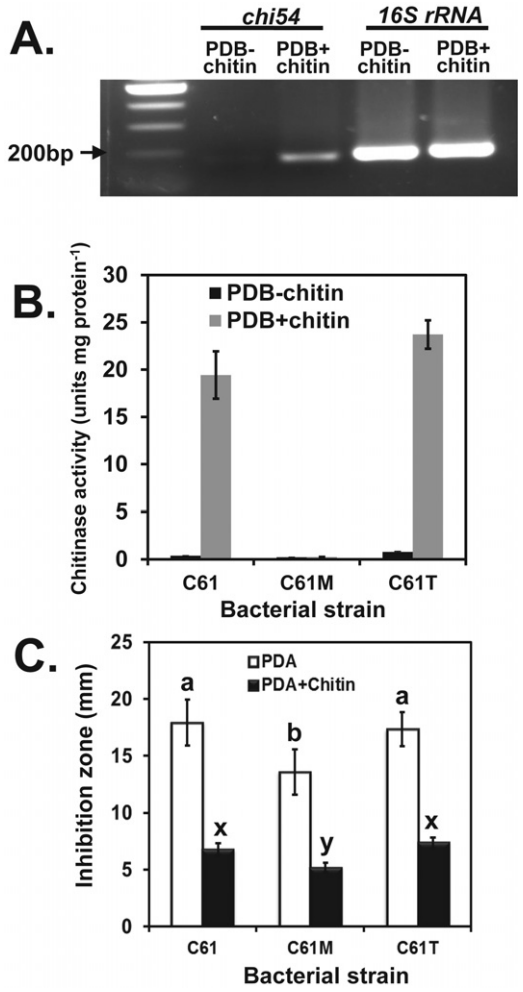

Fig. 3 Effect of chitin amendment on the production of extracellular chitinase activity and antifungal activities of *Chromobacterium* sp. C61. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the accumulation of transcripts from the *chi54* gene in *Chromobacterium sp.* C61. Total RNA was isolated from wild-type cells grown to the stationary phase [optical density at 600 nm ($OD_{600\ nm}$) > 2.4] in potato dextrose broth (PDB) with or without chitin. RT-PCRs were halted at the end of 24 cycles and the PCR products were loaded onto 1.5% agarose gel and visualized with ethidium bromide staining. Three independent experiments were conducted with similar results, and the data shown are from one experiment. (B) Chitinase activities of extracellular proteins partially purified from the growth of chitinase mutants and wild-type strains in PDB with and without colloidal chitin. (C) Antifungal activity against *Rhizoctonia solani* produced by inocula of *Chromobacterium* sp. C61, C61Chi54M and C61Chi54TS on potato dextrose agar (PDA) or a chitin-amended PDA plate. Data with different letters indicate that inhibition was significantly different ($P < 0.05$) based on Duncan's multiple range test.

sulphoxide (DMSO)-d_6. The ^1H and ^{13}C resonance assignments of the isolated compound are presented in Tables S1 and S2 (see Supporting Information). Proton resonances for most aliphatic hydrocarbon chain groups and all amino acid residues were assigned, using the nuclear magnetic resonance (NMR) cross peaks, to structures in the main and side chains. We named the isolated compound chromobactomycin based on the novelty of its structure, which contained one β-hydroxymyristate (HMS) group and nine amino acids (Fig. 5).

Time-of-flight mass spectrometry (TOF MS) analysis of chromobactomycin detected an $(M + H)^+$ peak at *m/z* 1196.6 with the predicted formula $C_{57}H_{86}N_{12}O_{16}$ (Fig. S4, see Supporting Information). A cyclic peptide was formed with a connection between the carboxyl group of Thr-2 and the amine group of Thr-9. Additional data for the structural analysis are presented in Figs S3–S10 (see Supporting Information). The MS^3 spectra of the compound yielded a fragment ion peak at *m/z* 868.4, which represented the loss of HMS and dihydroxybutyric acid (Dhb). A second fragmentation produced a mass at *m/z* 568.3, suggesting the loss of a glutamine–glycine–threonine moiety. Two main ions were detected (*m/z* 387.2 and 304.1) in a third fragmentation, and represented the loss of two threonines from the structure with *m/z* 568.3 and the loss of Dhb UT from the peak at *m/z* 387.2. The proposed structure for chromobactomycin, based on the NMR and MS analyses, is shown in Fig. 5.

Biocontrol activity of the purified chromobactomycin

Chromobactomycin, purified by preparative HPLC, when added to filter discs at 400 μg/disc, inhibited significantly the mycelial growth of *R. solani*, *Pyricularia grisea*, *B. cinerea*, *Alternaria longipes* and *C. gloeosporioides in vitro*. However, neither *Phytophthora capsici* nor *F. oxysporum* were inhibited in these assays (Table 1). There was no antibacterial activity against pv. *oryzae* (KACC10384), *Xanthomonas campestris* pv. *oryzae*, *Pseudomonas syringae* pv. *tomato* DC3000 or *Bacillus subtilis* QST713 (Table 1). Foliar applications of the partially purified chromobactomycin (50 μg/mL) reduced disease incidence in four pathosystems (Fig. 6).

The cell-free culture filtrate of the C61 strain had a surface tension of 72.7 mN/mL, which was similar to that of the water control. However, the surface tension-purified chromobactomycin was surface active, 42 mN/mL at 400 μg/μL. This finding indicates that the chromobactomycin produced by the C61 strain possessed weak surfactant activity.

Although the presence of chitin in the growth medium increased chitinase production, chromobactomycin production was reduced and correlated with lower *in vitro* biocontrol activity (Table 2). Based on the mass intensity value, chromobactin levels in the wild-type [$(326.67 \pm 24.63) \times 10^4$] and C61Chi54TS mutant [$(303.67 \pm 14.81) \times 10^4$] samples were higher than that for the C61Chi54M mutant [$(22.71 \pm 9.18) \times 10^4$]. One possibility for these differences in CLP levels could be variations in growth rate. Although the growth of the Chi54 mutants was similar to that of the wild-type in PDB or Luria–Bertani (LB) broth without chitin, growth of the C61Chi54M mutant had a lag phase of 6–9 h in PDB broth containing chitin (Fig. S11, see Supporting Information).

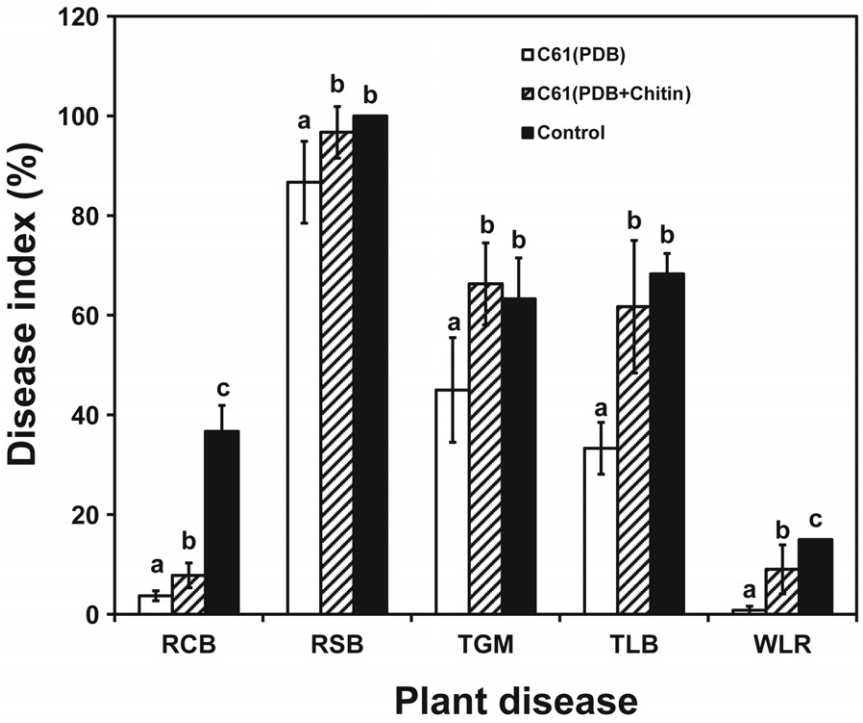

Fig. 4 *In planta* biocontrol efficacies of cell-free culture filtrates of *Chromobacterium* sp. C61 grown in the presence or absence of chitin. Applications were made by spraying intact plants with cell-free culture filtrates of C61 (1:3 dilution) grown in potato dextrose broth (PDB) or PDB + chitin; water was used as the negative control treatment. The disease index for each pathogen was based on the areas of the infected lesion. RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mould; TLB, tomato late blight; WLR, wheat leaf rust. Data are means ± standard deviations of three independent experiments with nine plants per treatment. Bars with different letters for each pathosystem have significantly different ($P < 0.05$) levels of disease based on Duncan's multiple range test.

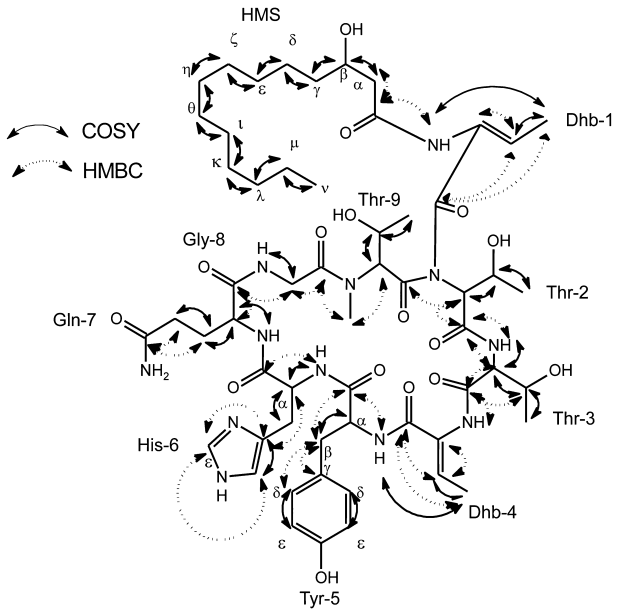

Fig. 5 Chemical structure correlations by correlation spectroscopy (COSY) and heteronuclear multiple-bond correlation spectroscopy (HMBC) of chromobactomycin**.** Solid arrows and dotted arrows refer to $^{1}H–^{1}H$ and $^{1}H–^{13}C$ correlations, respectively. Dhb, dihydroxybutyric acid; Gln, glutamine; Gly, glycine; His, histidine; HMS, β-hydroxymyristate; Thr, threonine; Tyr, tyrosine.

DISCUSSION

Because the suppression of plant disease results from multiple activities of biocontrol bacteria, it is important to determine which activities contribute the most to biological control under various conditions (Kim YC *et al*., 2011). Here, we demonstrate that the bacterium *Chromobacterium* sp. C61 produces and secretes both a chitinase and a novel CLP, which can contribute to biological control (Figs 2 and 5, respectively). Both lytic enzymes and CLP have been associated with biocontrol activity expressed by various microorganisms. Chitin is found in the cell walls of true fungi and in insect exoskeletons (Gooday, 1990), and amendment of soils with chitin has been correlated with increased populations of chitinolytic microorganisms (Wang *et al*., 2002). However, this is the first demonstration that both compounds actually contribute to the *in vitro* and *in planta* biocontrol activity noted by a single strain, i.e. C61.

Interestingly, the production of these two biocontrol-related compounds by C61 grown in liquid medium seems to be interconnected in some way. Specifically, the presence of added chitin induces an increase in Chi54 production (Fig. 3) and a concominant decrease in chromobactomycin. At this point, it is not clear how the production and/or secretion of the two products are regulated. Recently, we found that a Tn5 mutant in C61, which lacked antifungal activity, had a transposon insertion in a sequence identified as a *luxI*-encoding homoserine lactone synthase (H.J. Kim *et al*., unpublished results). Thus, it may be that Chi54 and chromobactomycin production are under quorum sensing control. Quorum sensing has been shown to regulate the expression of a major chitinase gene in *Chromobacterium violaceum* (Stauff and Bassler, 2011). Moreover, CLPs in other pseudomonads are also regulated by quorum sensing

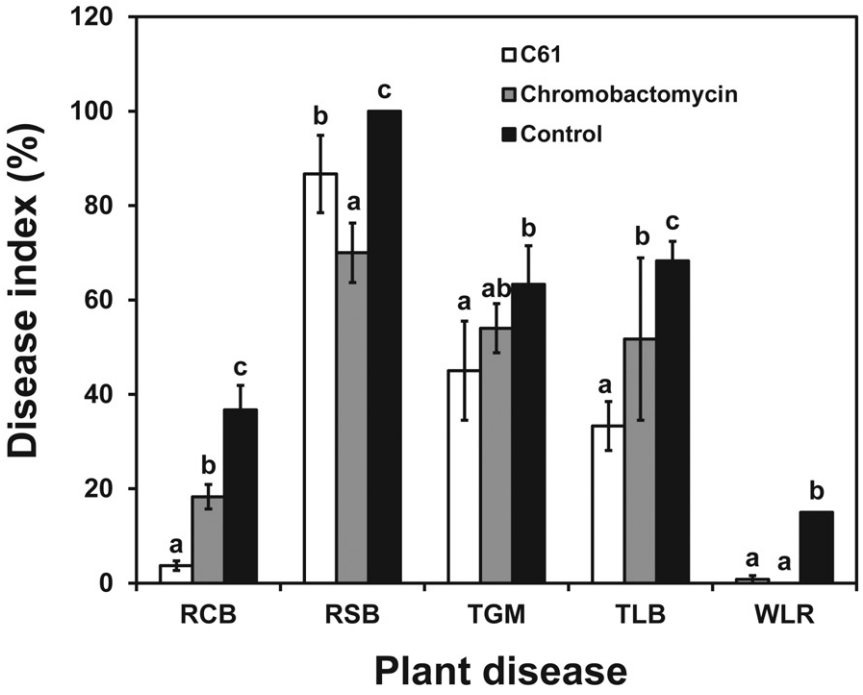

Fig. 6 *In planta* biocontrol efficacies of purified chromobactomycin or cell-free culture filtrates of *Chromobacterium* sp. C61. Applications were made by spraying intact plants with cell-free culture filtrates of C61 (1:3 dilution) and the partially purified chromobactomycin (50 μg/mL); water was used as the negative control treatment. The disease index for each pathogen was based on the areas of the infected lesion. RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mould; TLB, tomato late blight; WLR, wheat leaf rust. Data are means ± standard deviations of three independent experiments with nine plants per treatment. Bars with different letters for each pathosystem have significantly different ($P < 0.05$) levels of disease based on Duncan's multiple range test.

Table 1 *In vitro* activity of chromobactomycin against plant pathogenic fungi and bacteria.

Microorganism	Inhibition zone (mm)
Rhizoctonia solani (KACC40111)	6.0*
Pyricularia grisea (KACC40439)	6.0
Fusarium oxysporum (KACC40183)	–†
Phytophthora capsici (KACC40157)	–
Botrytis cinerea (KACC40573)	7.0
Alternaria longipes (KACC40028)	5.0
Colletotrichum gloeosporioides (KACC40003)	5.5
Xanthomonas campestris pv. *oryzae*	–
Pseudomonas syringae pv. *tomato* DC3000	–
Bacillus subtilis QST713	–

*Inhibition zone (mm) between the fungal mycelia or bacterial spreading and the edge of the paper disc treated with chromobactomycin (400 μg/disc). Data are the means of two independent experiments.
†No inhibition was detected.

(Raaijmakers *et al*., 2006). Consequently, the fact that growth of the mutant on chitin-amended medium demonstrated a long lag phase would correlate with reduced chromobactomycin levels, because of a delay in reaching a cell density threshold for quorum sensing to occur. Another possibility is that chromobactomycin production in the C61 strain is affected by metabolites from chitinase degradation, such as *N*-acetyl-glucosamine.

Although positively regulated by chitin (Monreal and Reese, 1969), negative regulation in the presence of glucose and *N*-acetyl-glucosamine in *Serratia marcescens* has been reported (Watanabe *et al*., 1997). Consequently, it seems that both environmental and nutrient conditions could be playing a role in the coordination of chitinase and CLP production in C61, as reviewed for other microbes by Fernando *et al*. (2005).

The CLP chromobactomycin from *Chromobacterium* sp. C61 contained an unusual amino acid, Dhb (2-amino-2-butenoic acid), which has been reported previously for microcystin heptapeptides (Sano and Kaya, 1995, 1998). Dhb is also present in other important CLPs in the tolaasin and syringomycin produced by pseudomonads (Raaijmakers *et al*., 2006). Dhb-1 connects the HMS and the cyclic peptide, possibly involving a thiotemplate mechanism, as discussed previously by Christiansen *et al*. (2008). The nonameric peptide of chromobactomycin is unique among the diverse CLP antibiotics produced by other bacteria (Ongena *et al*., 2007; Raaijmakers *et al*., 2006). Future research on chromobactomycin should involve larger scale fermentation and preparative extracts, so that the lethal dose 50% (LD_{50}) for various pathogens can be accurately determined and the nature of the resistance of some phytopathogens to this chemical can be evaluated.

Interestingly, CLPs play diverse roles in the ecology of plant beneficial bacteria (Raaijmakers *et al*., 2010). Many CLPs have biosurfactant activity and thus are important in colony spread, biofilm formation and root colonization on plant surfaces (Ongena and Jacques, 2007). The induction of systemic resistance against plant diseases has also been documented for particular CLPs (Jourdan *et al*., 2009; Ongena *et al*., 2007; Raaijmakers *et al*., 2010). Their antifungal activities are varied: orfamide and putisolvins inhibit zoospore motility and cause zoospore lysis, but have no effect on mycelial growth (de Bruijin *et al*., 2007; Kruijt *et al*., 2009). Weak biosurfactant activity was observed for

Table 2 Effect of a colloidal chitin amendment on the production of extracellular chitinase, production of chromobactomycin and *in vitro* antifungal activity of the *Chromobacterium* sp. C61.

Strain	Growth condition	Chitinase activity (U/mL/min)†	Concentration of chromobactomycin (mg/L)‡	Inhibition zone (mm)§	
				Rhizoctonia solani	*Botrytis cinerea*
C61	–Chitin	20.9 ± 0.7	88.2 ± 13.9	10.7 ± 0.6*	11.8 ± 1.1*
C61	+Chitin	59.8 ± 7.4*	60.8 ± 19.5	6.3 ± 0.6	7.5 ± 0.5

†Wild-type and mutants were grown in potato dextrose broth (PDB) with or without chitin for 3 days prior to the determination of chitinase activity using the fluorescent substrate 4-methylumbelliferyl-β-D-*N*,*N*-diacetylchitobioside in the culture filtrates. One unit (U) of chitinase activity was defined as the release of one micromole of 4-methylumbelliferone per minute per millilitre of culture filtrate.

‡The concentration of chromobactomycin in the culture filtrates of wild-type and mutants was determined by high-performance liquid chromatography (HPLC) using purified chromobactomycin as the authentic standard.

§Inhibited fungal growth of *R. solani* and *B. cinerea* by *Chromobacterium* sp. C61 was determined on potato dextrose agar (PDA) plates with or without colloidal chitin amendments.

Data are from three independent experiments and are represented as means and standard deviations. Asterisk indicates a significant difference between growth medium based on Student's *t*-test at $P < 0.01$.

chromobactomycin. Currently, we are examining the anticancer properties of chromobactomycin as reported for the CLP from *Chromobacterium violaceum* (Cheng *et al*., 2007). Studies of mutants impaired in the biosynthesis genes for chromobactomycin in C61 will be needed to confirm the relative importance of the antibiotic to chitinase activity in the biocontrol potential of this strain. The draft genome sequence of *Chromobacterium* sp. C61 indicated that a potential CLP operon exists (Kim HJ *et al*., 2011). Because biocontrol is multifactorial in nature (Kim YC *et al*., 2011), it will be interesting to investigate how the environmental factors affect the metabolome required for biocontrol. This work will contribute to the maximization of the utilization of biocontrol agents under field conditions.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The chitinolytic *Chromobacterium* strain C61, isolated previously from Korean soil (Park *et al*., 2005), was grown on nutrient broth (NB; Difco, Detroit, MI, USA) or LB medium (Sambrook *et al*., 1989) containing 50 μg/mL ampicillin and 0.2% colloidal chitin at 28 °C for routine culturing. Colloidal chitin was prepared as described previously (Park *et al*., 2007). *Escherichia coli* strains used in the genetic manipulations to obtain *Chromobacterium* sp. mutants were grown at 37 °C on LB medium (Sambrook *et al*., 1989). The medium was supplemented with kanamycin (50 μg/mL) for the growth of strains containing the plasmids pRL648 (Elhai and Wolk, 1988) and pCRII (Invitrogen Corporation, Carlsbad, CA, USA), with spectinomycin (50 μg/mL) for growth of a helper plasmid pRK2073 and tetracycline (12.5 μg/mL) for growth of the marker exchange vector pCPP54 (Miller *et al*., 1997). All strains were stored in 15% glycerol at −80 °C. The bacterial strains used to assess fungal antagonism were grown to an optical density at 600 nm (OD_{600nm}) in LB medium, centrifuged and the cells were suspended in sterile water to 1×10^8 colony-forming units (CFU)/mL.

Construction of *Chromobacterium* sp. C61 chitinase mutants

The cloning and subcloning procedures involved in the generation of the mutants were performed as described in Sambrook *et al*. (1989) and Ausubel *et al*. (1989). Plasmid DNA was isolated using an AccuPrep plasmid extraction kit (Bioneer Inc., Daejeon, South Korea), and genomic DNA was isolated by the cetyltrimethylammonium bromide (CTAB)-NaCl method (Sambrook *et al*., 1989). Restriction enzymes and modified enzymes were purchased from New England Biolabs (Ipswich, MA, USA).

A mutant defective in *chi54* gene expression was generated. The *chi54* gene harboured in plasmid pChi54 (Park *et al*., 2007) was disrupted by the insertion of a 0.9-kb *Bam*HI-digested fragment containing a kanamycin resistance gene from pRL648 into the unique *Bam*HI site within the gene (Elhai and Wolk, 1988). The interrupted *chi54* gene was exchanged with the chromosomal wild-type copy using the pCPP54 exchange vector, as described previously (Miller *et al*., 1997). Putative chitinase-deficient mutants were selected on plate medium containing 5% sucrose based on their resistance to kanamycin and their sensitivity to tetracycline.

A mutant, C61T, producing a more active chitinase, was generated by substitution of a tyrosine for a serine by modification of the *chi54* gene sequence (Park *et al*., 2007). Assessment of chitinase production involved the transfer of aliquots (5 μL) of the bacterial suspension onto 0.1% colloidal chitin–50% LB agar plates.

PCR analysis, using DNA extracted employing the standard CTAB method (Ausubel *et al*., 1989), was performed with *chi54*-specific primers (chi54F, 5'-GAACGCGTGCTGTTGTAAGA-3'; chi54R, 5'-GAATTGAGGAGG TCCGAATG-3') with Bioneer PCR premix to check for the presence of the interrupted gene in the *chi54* mutant and altered sequence in the T218S overactive mutant.

Chi54 transcript analysis

Chromobacterim sp. C61 cells were harvested from PDB cultures with or without 0.5% colloidal chitin at $OD_{600nm} = 0.7$ for mid-log phase cells. RNA was isolated using Trizol™ in accordance with the manufacturer's manual (Invitrogen Corporation). Reverse transcription (RT)-PCR was conducted

using the QuantiTect SYBR Green Reverse Transcription-PCR kit (Qiagen, Valencia, CA, USA). Two specific primers, forward (5'-AGAACAAGCG CCGCTACTAC-3') and reverse (5'-AGAACAAGCGCCGCTACTAC-3'), for the *chi54* gene, and specific primers for the 16S rRNA gene of *Chromobacterium* species, forward (5'-CCCACTGCCTCCCGTAAGGA-3') and reverse (5'-TGGCTCAGAACGAACGCTGG-3'), were used. A 25-μL mixture was incubated for 30 min at 50 °C for reverse transcription. Quantitative RT-PCR was performed using a Rotor-Gene 3000 Real Time Cycler machine (Corbett Research Inc., Sydney, Australia) for 40 cycles, with denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Quantitative RT-PCRs were halted at the end of 24 cycles and the PCR products were loaded onto 1.5% agarose gels and visualized with ethidium bromide staining.

Assessment of chitinase activity

Chromobacterium sp. strains were grown in PDB with or without 0.2% colloidal chitin at 28 °C for 48 h with agitation. Cells were removed from the cultures by centrifugation and chitinase activity was assessed in the culture supernatant. Enzyme activity was assayed by measuring the hydrolysis of 4-methylumbelliferyl-β-D-*N*,*N*-diacetylchitobioside (Sigma, St. Louis, MO, USA) in a reaction mixture (100 μL) containing 0.1 mM substrate and enzyme in 100 mM KH_2PO_4 buffer, pH 7.0. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 0.1 mL of 0.2 M Na_2CO_3, and the formation of chromogen was measured at an excitation wavelength of 360 nm and an emission wavelength of 440 nm in a Bio-Tek FLX-8000 fluorometer (Winooski, VT, USA). One unit of chitinase activity was expressed as micromoles of liberated 4-methylumbelliferone per minute per millilitre of culture supernatant or per milligram of purified chitinase. Activity was also assessed by observing the extent of the clearing zones on LB plates containing 0.2% colloidal chitin after transfer of test sample aliquots.

Partial purification of the extracellular proteins involved adjusting the supernatant to 50% ammonium sulphate with stirring at 4 °C for 24 h. The precipitated proteins were harvested by centrifugation at 7000 ***g*** for 30 min, and were then dissolved in 1 mL of 20 mM sodium acetate (pH 6.0) before storage at 20 °C. The protein concentration of each sample was determined by measuring the absorbance at 595 nm using the protein assay dye reagent method as described by the manufacturer (Bio-Rad Laboratories, San Francisco, CA, USA). The extracellular chitinase production from *Chromobacterium* sp. C61 wild-type and chitinase mutant was determined according to the method of Park *et al*. (2007). Briefly, the same amount of protein (20 μg), partially purified from the culture filtrates, was loaded onto SDS-PAGE gels. The proteins on the SDS-PAGE gels were stained with Coomassie blue for Western analysis, and their molecular weights were determined by comparison with low range SDS-PAGE standards (Bio-Rad Laboratories). The proteins were transferred to negatively charged nylon membranes (Roche Applied Science, Mannheim, Germany) by electroblotting and incubated at 4 °C for 24 h with a polyclonal antibody prepared previously against the purified Chi54 chitinase (Park *et al*., 2005). After washing twice with TBS-T buffer (20 mM Tris-HCl, pH 7, 100 mM NaCl, 0.5% Tween-20), the membrane was incubated with rabbit secondary antibody (Sigma) at room temperature for 1 h, followed by washing twice for 15 min with TBS-T and air drying. The chitinase bands were detected by exposing the membrane to Western Lighting™ Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer Inc., San Jose, CA, USA). In addition, the same amount of protein (20 μg), partially purified from the culture filtrates, was loaded onto SDS-PAGE gels, the proteins were separated and bands with chitinolytic activity were detected on the gels using 4-methylumbelliferyl *N*-acetyl-β-D-glucosamine [4-MU-$(GlcNAc)_2$].

Antifungal activity of the *Chromobacterium* sp. strain and the extracellular product

R. solani (KACC40111) and *B. cinerea* (KACC40573) were obtained from the Korea Agriculture Type Culture Collection Center (Suwon, South Korea). The pathogens were grown on PDA (Difco) at 28 °C. Suspensions of the *Chromobacterium* sp. C61 strains were spotted onto PDA plates prepared with or without colloidal chitin to measure antifungal activity, and agar discs (0.5 cm in diameter) bearing mycelia of each pathogen were placed in the centre of the plate. The antagonistic activities of the strains were evaluated by determining the extent of inhibited fungal mycelial growth compared with control plates lacking any bacterial inoculum. Cell-free filtrates were prepared from cultures grown on PDB containing 0.2% colloidal chitin for 3 days at room temperature with 200 r/min agitation to measure antifungal activities. An aliquot of the filtrate was boiled for 5 min to test the resilience of antifungal activity to heat. Both the control and heat-treated culture filtrates were transferred onto PDA plates to assess the inhibition of *R. solani* and *B. cinerea* growth, and onto plates with colloidal chitin to visualize chitinase activity.

Growth studies of the *chi54* mutant

Growth of the *chi54* mutant was compared with that of the wild-type in PDB with or without amendment with 0.2% colloidal chitin. The bacterial strains were grown at 25 °C with shaking at 200 r/min in PDB (Difco) for 24 h, and adjusted to $OD_{600\,nm} = 0.1$. Further growth was determined from serial dilutions by measuring CFU/mL growing on LB agar plates supplemented with appropriate antibiotics: 50 μg/mL ampicillin for the wild-type and 50 μg/mL kanamycin for the Chi54 mutants.

Assessment of *in vitro* antifungal activity of the purified Chi54

Inhibition of the fungal spore germination of *Chromobacterium* sp. C61 Chi54 by chitinase was examined using Chi54 generated from an *E. coli* strain containing the *chi54* gene in an expression vector, as described previously (Park *et al*., 2007). Four fungal pathogens, *C. gloeosporioides* (KACC4003), *B. cinerea* (KACC40573), *Cladosporium spharospermum* (KACC43548) and *F. oxysporum* (KACC40183), were obtained from the Korea Agriculture Type Culture Collection Center. Conidial suspensions (1×10^5 conidia/mL) of the fungal pathogens were prepared in sterile water from fungal cultures grown on PDA plates. Equal volumes of Chi54 (0.02, 0.2, 2 mg/mL) in 10 mM sodium acetate buffer (pH 6.0) or the buffer alone, and each fungal conidial suspension (1×10^3 conidia/mL), were mixed in a tube and incubated at 28 °C for 10 h. Germination of the fungal spores was observed under phase contrast microscopy with samples applied to a haemocytometer. The experiment was performed in duplicate.

Isolation of antifungal compounds

C61 was grown in 50 L LB medium with shaking at 27 °C for 36 h. Cells were removed by centrifugation at 10 000 ***g*** for 20 min and the supernatant was extracted twice with the same volume of ethyl acetate. The ethyl acetate extracts were dried in an evaporator at 45 °C and dissolved in a mixture of ethyl acetate–methanol (90:10, v/v). The extracts were loaded onto SPE (Supelco, Bellefonte, PA, USA) silica gel cartridge columns conditioned with an ethyl acetate–methanol (90:10, v/v) solvent system. The methanol concentration was increased by 10% to 100% for each elution step. The eluates were collected and concentrated to dryness in a rotary evaporator at 45 °C.

After measurement of the antifungal activities of the eluates, as described below, the active fractions were dissolved in methanol and injected into a prep-HPLC system equipped with a Dionex P680 model dual pump and a Dionex PDA-100 model photodiode array detector (Germering, Bayern, Germany) at 210 nm. The HPLC column was a μ-Bondapak C-18 stainless steel column (7.8 × 300 mm^2; film thickness, 10 μm). The solvent system was 85% (v/v) aqueous methanol, supplemented with 0.1% trifluoroacetic acid, and the flow rate was 2.0 mL/min. Each peak detected by HPLC analysis was collected and subjected to antifungal bioassays. All solvents used were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Instrumental analysis

A Varian model 500MS ion trap liquid chromatography (LC) mass spectrometer, connected to a 212-LC Binary LC system (Varian, Palo Alto, CA, USA) with a Prostar 335 photodiode array detector at 210, 260 and 280 nm, was employed to identify the isolated antifungal compound in the cultures. The HPLC column was a Chromsep C_{18} stainless steel column (150 × 2.0 mm^2). The mobile phase consisted of acetonitrile and ultrapure water containing 0.1% (v/v) formic acid, where the acetonitrile concentration was increased from 10% to 90% (v/v) over 30 min at a flow rate of 0.2 mL/min. The HPLC system was interfaced to an LC-MS^2 system equipped with electrospray ionization (ESI) interface in positive ion mode. The drying gas temperature and pressure were 300 °C and 20 psi, respectively. The capillary voltage was 80 V. Mass spectra were obtained in the *m*/*z* range 100–1500.

Mass spectra were obtained from a hybrid ion-trap TOF mass spectrometer (Shimadzu, Tokyo, Japan) equipped with an ESI source in positive ion mode at a mass resolution of 10 000 full width at half-maximum to calculate the exact mass. The analytical conditions were as follows: scan range, *m*/*z* 700–1500; spray voltage, +4.50 kV; detector voltage, 1.70 kV; skimmer voltage, 8.0 V; pressure of TOF region, 2.2×10^{-4} Pa; temperature, 40 °C; ion source temperature, 200 °C; trap cooling gas (Ar) flow rate, 94 mL/min; ion trap pressure, 1.8×10^2 Pa; collision gas (Ar) flow rate, 43 mL/min; ion accumulation time, 10 ms; precursor ion selected width, 3.0 *m*/*z* units; selected time, 50 ms; collision-induced dissociation collision time, 30 ms; collision energy, 100%; q_z = 0.251. MS^n data were acquired using the targeted function. The Shimadzu Composition Formula Predictor was also used to verify identifications. A Varian model Unity INFINITY plus 125-MHz NMR spectrometer and INOVA 500-MHz NMR spectrometer were used for the ^{13}C and ^{1}H analyses, respectively.

Determination of biosurfactant activity

Biosurfactant activity in cell-free culture medium was measured using a DuNouy model 3010 tensiometer (DuNouy, Tokyo, Japan), as described previously (Kim *et al*., 2007), and by the drop collapse assay using 10W-40 Penzoil (Penzoil Products Co., Houston, TX, USA), as described by Burch *et al*. (2010). The bacterial supernatants were prepared by centrifugation at 12 000 ***g*** for 10 min, and filtered through a 0.2-μm Millipore filter (Billerica, MA, USA) to remove residual bacterial cells. For surface tension measurement, the instrument was calibrated with air and water, giving a reading of 72.75 mN/mL. The surface tension measurement and drop collapse assay were conducted in three independent experiments with three replicates per sample.

Measurement of *in vitro* antifungal activity

Wild-type and mutant strain cells were grown in PDB with or without 0.2% colloidal chitin for 3 days to measure extracellular chromobactomycin production from C61. Chromobactomycin was extracted into ethyl acetate, fractionated by HPLC as described above and used in methanolic solutions for antifungal assays. The phytopathogenic fungal pathogens were *A. longipes* (KACC40028), *R. solani* (KACC40111), *P. grisea* (KACC40439), *F. oxysporum* (KACC40183), *Ph. capsici* (KACC40157), *B. cinerea* (KACC40573) and *Colletotrichum gloeosporioides* (KACC4003), each obtained from the Korea Agriculture Type Culture Collection Center. These pathogens were grown on PDA (Difco) at 28 °C. *Xanthomonas campestris* pv. *oryzae*, *Ps. syringae* pv. *tomato* DC3000 and *Ba. subtilis* QST718 were streaked onto fresh LB agar plates and incubated for 2 days at room temperature to measure the antibacterial activities of the purified chromobactomycin. The bacterial cells were harvested, resuspended in sterile water, adjusted to OD_{600nm} = 0.1 and 1 mL of the bacterial suspension was spread onto fresh LB agar plates. The CLP dissolved in methanol was added to sterile paper discs (8 mm in diameter; Advantec, Toyo Roshi Kaisha, Japan), dried and placed on the LB agar plates which were spread with the bacterial suspensions. The plates were incubated at 27 °C, and clear zones around the paper discs were assessed after 3 days.

The *Chromobacterium* sp. C61 strain inoculum was transferred to the edges of PDA plates to measure antifungal activity. Assays were also performed to measure mycelial growth inhibition on PDA plates with paper discs loaded with the purified materials. Methanol was used as a negative control. Sterilized paper discs (8 mm in diameter; Advantec) were treated with the isolated compound (400 μg/disc) dissolved in methanol. The paper discs were dried and placed at a distance of 2.5 cm from the pathogens provided as PDA plugs (5 mm in diameter) covered with growing fungal mycelia. The plates were incubated at 27 °C, and fungal growth was assessed after 5 days.

Plant disease suppression biocontrol assays

The biocontrol activity of *Chromobacterium* sp. strain C61 was tested *in planta* against six different plant pathogens (Kim *et al*., 2001), including *Magnaporthe oryzae* and *R. solani* on rice plants, *B. cinerea* and *Ph. infestans* on tomato plants, *Puccinia recondita* on wheat plants and

Colletotrichum coccodes on red pepper plants. The *in vivo* antifungal bioassays were conducted as described previously (Kim *et al*., 2001). Briefly, plants were grown in vinyl pots in a glasshouse at 25 ± 5 °C for 1–4 weeks. The plants were sprayed with 1:3 dilutions of cell-free culture filtrates of 3-day-old C61, C61Chi54M and C61Chi54TS grown in PDB with and without chitin, or the purified chromobactomycin (50 μg/mL) with Tween-20 as a wetting agent. Distilled water with Tween-20 was used as the negative control. The treated plant seedlings were inoculated with spores or mycelial suspensions from one of the six plant pathogens after 24 h, as described previously (Kim *et al*., 2001). Disease symptoms were rated 3–7 days after inoculation, depending on the pathogen. The pots were arranged in a randomized complete-block design, with three replicates per treatment, and each replicate consisted of nine plants. The disease index was determined by measuring the percentage of the infected leaf area. The experiment was conducted three times.

Statistical analysis

Data were subjected to analysis of variance using IBM SPSS Statistics version 19 (IBM Corporation, Somers, NY, USA). The differential effects of the bacterial treatments on disease severity were determined by Duncan's multiple range test ($P < 0.05$). The effect of a chitin amendment in the growth medium on the production of chitinase was evaluated using Student's *t*-test ($P < 0.01$).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Effect of the purified Chi54 protein on the germination rate of phytopathogenic fungal spores. Fungal spores of *Colletotrichum gloeosporioides* (Cg), *Botrytis cinerea* (Bc), *Cladosporium spharospermum* (Cs) and *Fusarium oxysporum* (Fo) were treated with different concentrations of the purified Chi54 protein in 10 mM sodium acetate buffer (pH 6.0) or the buffer without the Chi54 protein. The germination rate was measured by counting the number of fungal spores that germinated versus the total number of spores using a haemocytometer under a phase contrast microscope 10 h after incubation at 28 °C. The data are the means of two independent experiments and the vertical bars indicate the standard deviations. Different letters indicate significant differences between fungal pathogens according to Duncan's multiple range test ($P < 0.05$).

Fig. S2 High-performance liquid chromatograms of ethyl acetate-extracted compounds from cell-free filtrates of *Chromobacterium* sp. C61. The solvent system was 85% (v/v) aqueous methanol supplemented with 0.1% trifluoroacetic acid at 2.0 mL/min, and the peaks were detected at 210 nm. The chromobactomycin peak from *Chromobacterium* sp. C61 is shown with an arrow, and this material was used to obtain the time-of-flight mass spectrum provided.

Fig. S3 Ion trap-time-of-flight mass spectrometry (IT-TOF MS), MS^2 and MS^3 data of the isolated antifungal compound.

Fig. S4 Correlation spectra of dihydroxybutyric acid (Dhb) in methanol-d_6 of the isolated compound.

Fig. S5 Heteronuclear multiple-bond correlation spectra of dihydroxybutyric acid (Dhb) and Tyr-5 in methanol-d_6 of the isolated compound.

Fig. S6 Heteronuclear multiple-quantum correlation spectra of β-hydroxymyristate in methanol-d_6 of the isolated compound.

Fig. S7 Heteronuclear multiple-bond correlation spectra in methanol-d_6 identifying all carbonyl groups in the amino acid residues of the isolated compound.

Fig. S8 Heteronuclear multiple-bond correlation spectra of Tyr-5 in methanol-d_6 of the isolated compound.

Fig. S9 Connectivities observed in correlation and heteronuclear multiple-bond correlation spectra, leading to sequence assignment of the isolated compound.

Fig. S10 The proposed modifications of chromobactomycin structure identified by tandem mass spectrometry (MS^2). Dhb, dihydroxybutyric acid; Gln, glutamine; Gly, glycine; His, histidine; HMS, β-hydroxymyristate; 3-HTDA, 3-hydroxytetradecanoic acid; Thr, threonine; Tyr, tyrosine.

Fig. S11 Effects of the *chi54* mutations on aerobic growth in the presence of chitin. Cultures were grown aerobically in potato dextrose broth (PDB) with 0.2% chitin, and the growth of bacterial strains was determined by measuring the colony-forming units/mL (CFU/mL) of serial diluted cultures. Bacterial populations were assessed at each time point using analysis of variance (ANOVA), and mean CFU/mL values were compared according to Duncan's multiple range test ($*P < 0.05$ and $**P < 0.01$). The data are the means of three independent experiments and vertical bars indicate standard deviations.

Table S1 ^1H chemical shift assignments of chromobactomycin in methanol-d_4 (A) and dimethylsulphoxide (DMSO)-d_6 (B).

Table S2 ^{13}C chemical shift assignments of chromobactomycin in methanol-d_4 (A) and dimethylsulphoxide (DMSO)-d_6 (B).