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Escherichia coli Can Produce Recombinant Chitinase in the Soil to Control the Pathogenesis by *Fusarium oxysporum* Without Colonization

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Abstract *Fusarium* wilt of cucumbers was effectively controlled by *Escherichia coli* expressing an endochitinase gene (*chiA*), and the rate was as effective (60.0%) as the wild-type strain *S. proteamaculans* 3095 (55.0%) where the gene was cloned. However, live cells of soil inoculated *E. coli* host harboring the *chiA* gene did not proliferate but declined 100-fold from 10^8 CFU during the first week and showed less than 10 cells after day 14, suggesting that *E. coli* was able to express and produce the chitinase enzyme to the soil even as the population was gradually decreasing. Because the majority of the strains was alive for only a short period of time and the *Fusarium*-affected seedlings showed symptoms of wilting within 7–10 days, it seems that the pathogen control was decided early after the introduction of the biocontrol agent, eliminating the survival of the antagonist. These results indicated that soil inoculated *E. coli* could sufficiently express and produce the recombinant protein to control the pathogen, and root or soil colonization of the antagonist might not be a significant factor in determining the efficacy of biological control.

Keywords: *Escherichia coli*, chitinase, biocontrol, colonization, *Fusarium* wilt

Production of hydrolytic enzymes such as chitinase to control the propagation of plant pathogens is one of several biocontrol mechanisms exhibited by biocontrol plant growth promoting bacteria [17]. Many of these bacteria are shown to have multiple chitinase enzymes [7, 26] that are produced either by separate genes [8, 29] or by proteolytic cleavage of a larger molecule to make genetically related species [24, 28]. These multiple enzyme systems are likely to enable the bacteria to perform efficient hydrolysis of chitin in the decomposition of the biopolymer, and subsequently be used as a carbon and energy source. The chitinase genes were cloned and characterized in different variants of *Serratia marcescens*, and several varieties were reported in the literature; *chiA* [2, 14, 29], *chiB* [1, 8, 11, 29], *chiC* [26], and another novel chitinase gene [9].

In determining the role of chitinase in the control of phytopathogens, laboratory experiments with purified chitinase from *Serratia marcescens* showed that the enzyme alone was effective in the control of *Sclerotium rolfsii* [23]. Other researchers have indicated that crude preparation of chitinase from *Aeromonas caviae* [13], *Enterobacter agglomerans* [4], *Pseudomonas fluorescens* [22], and *Serratia plymuthica* [7] were also highly effective in the control of various soilborne plant pathogens. Purified enzymes, however, have limitations in applying to the real farming situation owing to high cost in purification of the enzyme and inconvenience in distribution and storage of the enzyme product. Therefore, developing an enzyme product for agricultural use may require extensive research on the formulation to cut down on price and to achieve the proper stability of the enzyme.

An easy alternative method to deliver the enzyme is to use a beneficial bacterial host, and many of the natural antagonists isolated from soil have been suggested to be applied as biocontrol agents [5]. However, regulating or continually providing certain effective enzymes until the pathogen is controlled is not always easy because of the unknown genetics of many natural hosts. On the other hand, a biocontrol negative background *Escherichia coli* can be a convenient host to actively provide the antagonistic enzyme by using the well-known recombinant DNA techniques in this organism. Furthermore, expressing one biocontrol mechanism by cloning one specific biocontrol enzyme will give information to investigate the *in vivo* effectiveness of certain enzymes alone in the control of plant pathogens. It is, however, suspicious whether *E. coli* can survive long enough to produce a sufficient amount of the recombinant enzyme to control the pathogen in the soil where the

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amount of nutrients and temperature conditions are not met to the growth requirements of the strain.

To better understand whether *E. coli* cells are able to properly control the phytopathogens, a chitinase gene previously cloned from *Serratia proteamaculans* 3095 [16] was sequenced in this study and the endochitinase-type nature of the enzyme verified; the chitinase gene, denoted *chiA*, was subsequently used to transform *E. coli* LE392 with no biocontrol activity. The efficacy of pathogen control exerted by recombinant *E. coli* at 25–28°C was investigated by artificially induced *Fusarium* wilt in cucumbers to see the effectiveness of *E. coli* in producing the recombinant enzyme in the soil. To know whether the amount of enzyme produced was comparable to the natural environmental strain, the suppression rate was compared with that determined for the mother strain *S. proteamaculans*. Survival of the recombinant strain in the soil system was also determined to investigate the influence of the colonization of biocontrol agents on the efficacy of biological control in the soil.

MATERIALS AND METHODS

Strains, Growth Medium, Growth Condition, and Materials for Biocontrol Experiments

Serratia proteamaculans 3095 was originally isolated from soil in Gyeongju A-Hwa (Gyeongnam) area as an antagonist of *Fusarium oxysporum*, which causes *Fusarium* wilt in egg plants (*Solanum melongena* L.) [17]. Cells were grown in Luria Bertani (LB) medium [25] at 25°C. The *Escherichia coli* strain for the amplification of recombinant plasmids was either DH5α or JM109, and the host for assaying the efficacy of chitinase enzyme in biocontrol experiments was LE392. *Escherichia coli* SM17 (pUT/Km) containing the miniTn5Km DNA fragment [12] was kindly provided by Dr. D. P. Roberts at USDA-ARS (Beltsville, MD, U.S.A.). *E. coli* was grown in LB medium at 37°C. Antibiotics of the following concentrations were added to the medium when needed; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. Colloidal chitin for chitin-agar plates was prepared by the procedure described in Lee [16] but with the following modifications; about 15 g of chitin powder (Sigma, C-7170) was acid digested overnight at 4°C by adding 150 ml of concentrated HCl. The mixture was then passed through Whatman No. 1 paper, and the colloidal chitin in the run-through fraction was washed with distilled water, 3–4 times, employing centrifugation at 1,000 ×g for 10 min. The final pH was adjusted to 7 with KOH. Colloidal chitin was centrifuged and resuspended in 0.06 M phosphate buffer (pH 7.5) and left at 4°C until use. Colloidal chitin preparation was centrifuged and the wet-weight amount was incorporated to chitin-agar plates. The composition of the chitin-agar plates was prepared with 1% colloidal chitin, following the published method [17]. Plant pathogen *Fusarium oxysporum* f. sp. *cucumerinum* KACC 40527 was kindly provided by the Korean Agriculture Culture Collection at the National Institute of Agriculture Biotechnology (Suwon, Gyeonggido). The pathogen was used to artificially induce *Fusarium* wilt in cucumbers (see Biocontrol Assay below).

The artificial soil employed was TKS 2, purchased from Korea Agriculture Materials Ltd. (Seoul, Korea). All the soil in the experiments was used as it was and not autoclaved. Cucumber seeds (*Cucumis sativus* LINN; Summer Long) used in the biocontrol experiment of *Fusarium* wilt were purchased from Seminis Korea Inc.-Choongang Seeds. (Seoul, Korea). Young plants were cultivated in the laboratory, having a temperature fluctuation between 25–28°C to mimic the temperature fluctuation of the environment. Fluorescent light was left on for 24 h.

Transformation

Escherichia coli DH5α or LE392 was grown in 100 ml of TYM [25] until the mid-exponential phase (OD600=0.5) and harvested by centrifugation at 1,000 ×g for 10 min. Competency was generated by the method of Hanahan, described in Sambrook and Russell [25]. Transformants were selected on LB agar plates containing appropriate antibiotics.

DNA Isolation

Quick isolation of recombinant plasmids from *E. coli* DH5α was performed by the alkaline lysis method described in Sambrook and Russell [25]. Plasmid preparation for DNA sequencing reactions was done using the Wizard Plus Minipreps DNA Purification System (Promega, WI, U.S.A.). Total chromosomal DNA was isolated from *S. proteamaculans* 3095 using the Wizard Genomic DNA Purification Kit (Promega, WI, U.S.A.). DNA fragments separated on agarose gels were purified using the Geneclean II Kit (Bio 101, CA, U.S.A.).

Southern-Blot Hybridization

About 3–5 μg of genomic DNA was electrophoresed on 0.8% agarose gel and transferred onto nylon membranes (Schleicher and Schuell, Germany) by capillary action using 10× SSC as the transfer buffer [25]. DNA on nylon membrane was fixed by baking the membrane at 80°C for 2 h, before hybridization experiments. Prehybridization and hybridization conditions were as those described by Bryant and Tandeau de Marsac [3]. The hybridization probe was labeled with either [α-^{32}P]-dCTP or digoxigenin-dUTP using a random priming kit (Roche, Germany). Positive signals for digoxigenin-dUTP-labeled probes were identified with a chemiluminescent detection kit following the procedure recommended by the manufacturer (Roche, Germany).

Polymerase Chain Reaction and Cloning of *chiA*

PCR primers, 5' GCGAAGTGGTCGGTACTTACTTCG-ACGATGT 3' and 5' TGCAGTAA ACGGCGTTGCCGG-ATACGAT 3', were designed by Lee [16] by analyzing the most conserved sequence of the published chitinase A gene sequence of *Serratia marcescens* 2170 [29]. Oligonucleotides were synthesized from Bioneer Corp. (Daejeon, Korea). The polymerase chain reaction (PCR) was performed with Taq polymerase (Takara, Japan) using the Minicycler by MJ Research (Waltham, MA, U.S.A.). These primers gave a 730-bp band in PCR, and the PCR product gave a 2.6-kb EcoRI-HindIII band signal on Southern-blot hybridization experiment. The 2.6-kb EcoRI-HindIII fragment was subsequently cloned by screening the partial plasmid library contained in *E. coli* JM109; the transformant with the 2.6-kb DNA fragment excreted chitinase to the medium, which caused the formation of a clear zone on colloidal chitin-agar plates. This positive plasmid was denoted pLC66.

DNA Sequencing of *chiA* Gene

The nucleotide sequence of pLC66 was revealed by using the sequencing service of Macrogen Inc. (Seoul, Korea). To determine the full gene sequence of chitinase A gene, a restriction map of pLC66 was generated and various subclones having different insert sizes were constructed; both strands of the insert DNA were sequenced using M13 forward and reverse primers. The nucleotide sequence file has been deposited in GenBank (Accession Number DQ285568).

Interposon Mutagenesis of *chiA* Gene in *Serratia proteamaculans* 3095

The 1.3-kb KspI fragment from the full *chiA* gene was deleted and the 3' overhangs were digested with T4 polymerase (Promega, WI, U.S.A.). The resultant DNA was ligated with the 2.3-kb EcoRI miniTn*5*Km gene that was produced by digesting pUT/Km with EcoRI; the miniTn*5*Km gene was also made blunt by filling in the 5' overhangs with Klenow enzyme of DNA polymerase I. Total ligation mixture was used to transform DH5α. Two plasmids, pLC700 in which the kanamycin resistant marker was inserted in the same transcriptional orientation as *chiA*, and pLC701 in which it was inserted in the opposite orientation, were selected by kanamycin resistance. The *DchiA*::Tn*5* mutant constructs were verified by restriction enzyme mapping.

Biocontrol Assay

To artificially induce *Fusarium* wilt in cucumbers, 10^7 spores in 40 ml of regular tap water were mixed with 200 ml of soil, and 5 cucumber seeds were planted using 9 cm×8 cm (diameter×depth) pots. Seeds were washed with soap and water before sowing to remove any residual chemicals that might have been coated on the surface. Biocontrol assay methods for *Phytophthora* blight in peppers were from Chung and Kim [5]. The biocontrol strain of 10^8 cells, washed and resuspended in 10ml of water, was introduced 1 day after sowing the cucumber seeds or planting the pepper seedlings. Survivals were counted 12 days after the introduction of the biocontrol agents. To calculate the disease suppression rates, survivals out of 5 seeds or seedlings were counted with 8 repetitions. Analysis of variance (ANOVA) was performed, and differences between means of treatments were determined by Duncan's test using SAS ver. 9.1.

Determination of Survival Rate of *Escherichia coli* in the Soil

Pots (9 cm×8 cm, diameter×depth) were prepared with 200 ml of soil, and 5 cucumber seeds were planted in each pot. The artificial soil with endogenous microbial community was used as it was, and not autoclaved. One day after sowing, pots were treated with 10^8 CFU of *E. coli* LE392 or *E. coli* LE392 (pLC66) washed and resuspended in 10 ml of sterile water. Controls were treated with just 10 ml of sterile water. Pots were watered with regular tap water every 2 days with a water sprayer. Surviving bacterial cells in 200 ml of soil were counted every 7 days until day 35, by dilution plating. Since *E. coli* LE392 was able to form colonies at 41°C without any significant decrease in numbers when the colony counts were compared with those on the plates incubated at 37°C, the majority of the endogenous soil bacteria on the dilution plates were suppressed by incubating the plates at a higher temperature of 41°C. Moreover, introduced *E. coli* cells were readily distinguishable from the endogenous bacteria even if other strains were still growing at 41°C. The survival of both *E. coli* LE392 host only and *E. coli* LE392 (pLC66) was monitored by cell counts on LB and LB containing ampicillin plates, respectively. The survival curve of the live cells was generated by employing means of two independent experiments.

RESULTS

Cloning of *chiA* from *Serratia proteamaculans* 3095

Southern-blot hybridization experiments using the 730-bp PCR product as a probe gave a hybridization signal of the 2.6-kb EcoRI-HindIII fragment with the genomic DNA of *Serratia proteamaculans*. The plasmid containing the cloned chitinase gene, *chiA*, was denoted pLC66. To determine the full gene sequence of the cloned 2.6-kb EcoRI-HindIII fragment containing the *chiA* gene, subclones were generated in either pUC19 or pBluescript SK$^-$; both strands of the subclones were sequenced. Analysis of the sequence revealed that the gene was approximately located in the middle of the cloned fragment (Fig. 1). The deduced

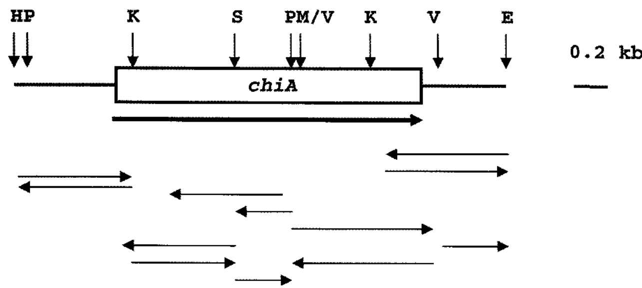

Fig. 1. Physical map of *chiA* from *Serratia proteamaculans* 3095.
Vertical arrows indicate the position of restriction enzyme sites and horizontal arrows indicate the sequencing strategy; plain arrows indicate sequence data obtained from subclones with either the M13 forward or reverse primers. The bold arrow underneath the gene indicates the direction of the transcription. Abbreviations for restriction enzyme: E, EcoRI; H, HindII; K, KspI; M, SmaI; P, PstI; S, SalI; V, EcoRV.

amino acid sequence indicated that the gene would encode a 563-amino-acid endochitinase type enzyme. The amino acid sequence was 99.3% and 99.1% identical to the chitinase A reported in *S. marcescens* 2170 [29] and *S. marcescens* BJL200 [2], respectively; only 4 to 5 positions were different and the differences in the amino acids were conservative substitutions (results not shown). By comparison with the mature protein studied in *S. marcescens* 2170, the 563 presequence in *S. proteamaculans* also seems to be processed after the 23rd alanine to produce the mature enzyme with only 540 amino acids. The calculated molecular mass was 58.5 kDa, suggesting that *chiA* could be the corresponding gene for the purified chitinase protein from *S. proteamaculans* 3095, which had been determined to be 62 kDa by SDS-PAGE analysis [18].

Biological Control of *Fusarium* Wilt by *Escherichia coli* Producing the Chitinase A of *Serratia proteamaculans* 3095

To investigate the *in vivo* chitinase activity on biological control of phytopathogenic fungi, the *chiA* gene was introduced into *Escherichia coli* having no chitinase background. As shown in Fig. 2D, *E. coli* LE392 harboring the *chiA* gene (pLC66) of *S. proteamaculans* 3095 produced and excreted a significant amount of chitinase into the medium, which caused a clear zone on the colloidal chitin-agar plate. However, *E. coli* LE392 alone (Fig. 2B), *E. coli* LE392 harboring vector only (pUC19) (Fig. 2C), or the interposon mutant of *DchiA*::Tn*5* (pLC700) (Fig. 2E) did not form visible clear zones, suggesting that these strains could not produce any active chitinase. This indicated that the *chiA* gene on pLC66 was transcribed and translated, and possibly processed in *E. coli* to give the functional chitinase enzyme.

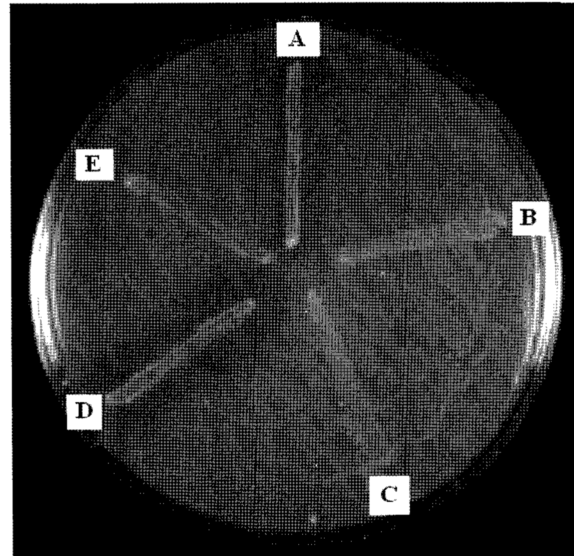

Fig. 2. Clear zone formation on colloidal chitin-agar plate by *E. coli* LE392 with and without the *chiA* gene.
A. *Serratia proteamaculans* 3095; **B**. *E. coli* LE392; **C**. *E. coli* LE392 (pUC19); **D**. *E. coli* LE392 (pLC66); **E**. *E. coli* LE392 (pLC700).

The biocontrol efficacy of *E. coli* producing chitinase on *Fusarium* wilt was determined in an artificial system using

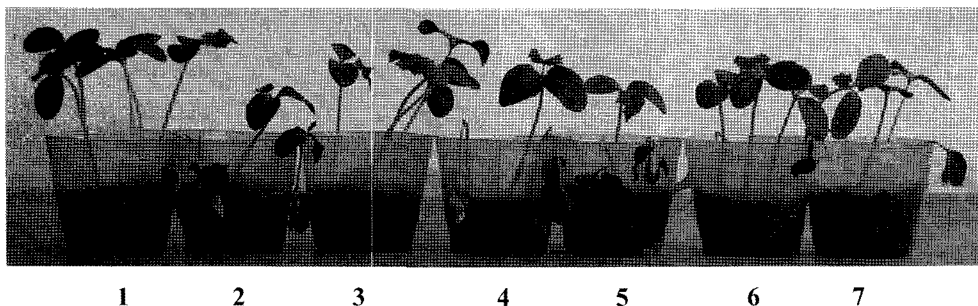

Fig. 3. A picture of *Fusarium* wilt suppression in cucumbers by *Serratia proteamaculans* 3095 and *E. coli* LE392 harboring the *chiA* gene.
Lane 1, No pathogen (5 survived); lane 2, Pathogen Only (1 survived); lane 3, Pathogen+*S. proteamaculans* 3095 (4 survived); lane 4, Pathogen+*E. coli* LE392 (1 survived); lane 5, Pathogen+*E. coli* LE392 (pUC19) (1 survived); lane 6, Pathogen+*E. coli* LE392 (pLC66) (4 survived); lane 7, Pathogen+*E. coli* LE392 (pLC700) (2 survived).

9 cm×8 cm (diameter×depth) pots holding 200 ml of soil and 5 cucumber seeds. Within 4 days, the cucumber seeds sprouted, and most of the seedlings in the pathogen-only control pots showed symptoms of wilting of around 7–10 days after sowing. The diseased seedlings eventually died in every case investigated. Fig. 3 shows the growth pattern of the cucumbers, and 3–5 seedlings out of 5 planted seeds in each pot that received *E. coli* LE392 (Fig. 3, lane 4) or *E. coli* LE392 (pUC19) (Fig. 3, lane 5) were affected by *Fusarium* wilt. Seedlings in pots that received *E. coli* LE392 (pLC66) (Fig. 3, lane 6), however, supported robust growth of cucumber seedlings, in spite of the presence of *Fusarium oxysporum* spores; the survival rate of the seedlings was comparable to that of the seedlings in pots treated with the wild-type *S. proteamaculans* 3095 (Fig. 3, lane 3). This phenomenon could be reversed if the *chiA* gene on pLC66 was deleted and inactivated with a transposon (Fig. 3. lane 7). Whereas the overall suppression rates of *Fusarium* wilt were in the 30's for the pots treated with *E. coli* LE392 host only, *E. coli* LE392 containing the vector only (pUC19), or *E. coli* LE392 with *ΔchiA*::Tn*5* (pLC700), the disease prevention rate of the pots treated with the chitinase producing *E. coli* LE392 (pLC66) was 60.0% (Table 1). This number was comparable to the suppression rate achieved by treating the pots with the wild-type strain *S. proteamaculans* 3095.

To show that the biocontrol seen in Table 1 is not due to some plant immunity, the same experiments were conducted using *Phytophthora capsici* having non-chitinacious zoospores as pathogen. Table 2 shows that *Phytophothra* blight was not preventable even with the *E. coli* having the *chiA* gene. This indicated that the biological control of *Fusarium* wilt by *E. coli* LE392 (pLC66) was truly due to the *E. coli* produced chitinase enzyme.

Table 1. Disease suppression rates of *Fusarium* wilt in cucumbers by *Escherichia coli* harboring the *chiA* gene from *Serratia proteamaculans* 3095.

Treatment[a]	Survivals[b]	Suppression rate (%)
No pathogen	4.88 A	
Pathogen only (10^7 spores/pot)	1.50 C	30.0
Pathogen+*S. proteamaculans* 3095	2.75 B	55.0
Pathogen+*E. coli* LE392	1.88 C	37.6
Pathogen+*E. coli* LE392 (pUC19)	1.75 C	35.0
Pathogen+*E. coli* LE392 (pLC66)	3.00 B	60.0
Pathogen+*E. coli* LE392 (pLC700)	1.63 C	32.6

[a]Bacterial cells of 10^8 were resuspended in 10 ml of water and poured into pots (9 cm×8 cm, diameter×depth) containing 200 ml of soil and 5 cucumber seeds, 1 day after the pathogen (*Fusarium oxysporum*) inoculation. Survivals were counted 12 days after the introduction of the bacteria.
[b]Number of surviving seedlings out of 5 planted seeds. Values are the means of 8 independent pots. Treatments within an experimental trial followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Duncan's test.

Table 2. Disease suppression rates of *Phytophthora* blight in peppers by *Escherichia coli* harboring the *chiA* gene from *Serratia proteamaculans* 3095.

Treatment[a]	Survivals[b]
No pathogen	5.00 A
Pathogen only (2×10^3 zoospores/pot)	1.13 C
Pathogen+*S. proteamaculans* 3095	3.13 B
Pathogen+*E. coli* LE392	1.63 C
Pathogen+*E. coli* LE392 (pUC19)	1.63 C
Pathogen+*E. coli* LE392 (pLC66)	1.50 C
Pathogen+*E. coli* LE392 (pLC700)	1.50 C

[a]Bacterial cells of 10^8 were resuspended in 10 ml of water and poured into pots (9 cm×8 cm, diameter×depth) containing 200 ml of soil and 1 pepper seedling, 1 day after the pathogen (*Fusarium oxysporum*) inoculation. Survivals were counted 12 days after the introduction of the bacteria.
[b]Number of surviving seedlings out of 5 seedlings. Values are means of 8 independent experiments. Treatments within an experimental trial followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Duncan's test.

Survival of *Escherichia coli* LE392 Harboring the *chiA* Gene in the Soil System

The survival rate of *chiA*-harboring *E. coli* was monitored for 35 days by counting the survivors in the rhizosphere of cucumber seedlings. Cucumber seeds were planted to mimic and provide the natural environment where rhizospheric bacteria proliferate. Control pots received sterile water. As indicated in Fig. 4, live cells of *E. coli* LE392 (pLC66) decreased more than 100-fold only after 7 days, and less than 10 colonies were detected on day 14 (closed circles); none were measured afterwards. A similar survival rate was detected with the host-only control pots (Fig. 4, open circles). Control pots that did not receive bacteria were negative for the *E. coli* in question throughout the experiment (results not shown). These results indicated that *E. coli* LE392 cells could not

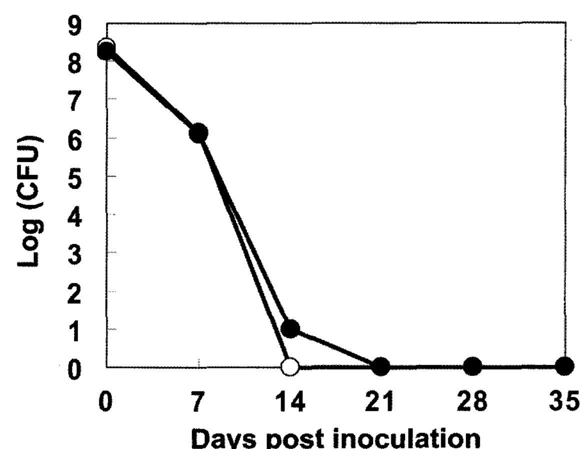

Fig. 4. Survival of *Escherichia coli* LE392 and *E. coli* LE392 harboring *chiA* in the soil.
Values are means of 2 independent experiments. Each pot received 200 ml of soil, 5 cucumber seeds, and 10^8 CFU of *E. coli* LE392 (open circles) or *E. coli* LE392 (pLC66) (closed circles). Error bars are omitted for clarity.

survive more than 2 weeks in the soil system employed, but was able to express the recombinant plasmid and sufficiently produced chitinase during this short survival to prevent the propagation of *F. oxysporum* spores with an efficacy comparable to that of the natural strain *S. proteamaculans*.

DISCUSSION

Mass production and industrial application of purified chitinase enzymes are being investigated to control crustacean waste at the seafood processing sites [10, 15], but agriculture usage of purified enzymes to control plant pathogens does not have commercial advantages because of the high cost in manipulating the enzyme product. Instead of using the expensive purified enzyme, we conducted a research using *E. coli* as a cheap and convenient delivery host for the enzyme. For this purpose, a biocontrol-negative *E. coli* host was transformed with a plasmid having the *chiA* gene. Because the *chiA* gene on the original cloned 2.6-kb EcoRI-HindIII DNA fragment (Fig. 1) contained its natural σ^{70}-type promoter besides the *lac* promoter provided by the pUC19 vector, the expression of *chiA* seemed to have been enhanced, as suggested by a large production of the chitinase enzyme by the recombinant *E. coli* (compare the clear zone on Fig. 2D with 4A). The recombinant *E. coli* used in the suppression of *Fusarium* wilt showed biocontrol rate that was as effective as the mother strain, *Serratia proteamaculans* (Table 1 and Fig. 3). The a biocontrol activity seems to be truly due to produced chitinase from *E. coli* in the soil, as the same strain could not prevent the propagation of non-chitinacious pathogen (Table 2). Because the strains were washed with water and diluted to have 10^8 CFU/ml, the biocontrol activity shown in Fig. 3 is not due to carried-over enzyme produced in the growth phase, but was truly produced after the *E. coli* had been inoculated in the soil system. Moreover, *E. coli* was able to produce the chitinase as the population was gradually dying (Fig. 4), indicating that active proliferation or colonization of the host does not seem to be required to produce a sufficient amount of enzyme to effectively control the pathogen to the level seen with the natural wild-type strain (Table 1). Since the live cells of recombinant *E. coli* were in the 10^6 cells only a week or so and the symptoms of wilting showed within 7–10 days of sowing the seeds to lead to eventual death (Fig. 3), it can be deduced that the pathogen was controlled early in the pathogenesis in a all-or-none fashion. It is generally accepted that biocontrol agents need to proliferate and colonize in the 10^5 CFU range in the area in question for some time to exert efficient biological control [19]. With chitinase-producing strains, however, efficient colonization or continuous survival of biocontrol agents might not be a prerequisite, especially when the fungal pathogens could be prevented early in the pathogenesis.

The results in this study indicate that *E. coli* can also be an effective biocontrol agent when the proper gene for the biocontrol mechanism is provided by recombinant DNA technology. When it comes to applying to the real environment, however, concerns do exist with releasing *E. coli* and the recombinant gene to the environment. However, even when $1-3\times10^8$ cells were inoculated on the soil, the survivals were limited so that less than 10 cells were found after 14 days (Fig. 4). A similar decrease in *E. coli* cell counts on nonselective plates (Fig. 4, opened circles) or the ones harboring the recombinant plasmid on selective plates (Fig. 4, closed circles) suggests that loss of plasmid in a nonselective condition is not a factor in the rapid decrease in the survival of the cells with the chitinase gene, especially when the cells are not proliferating. The short survival rate, therefore, is likely due to the innate characteristics of *E. coli* under the condition. This low survival of *E. coli* suggests that recombinant strains are not likely to cause large impacts on the environment. Another researcher also found that the externally introduced DNA was relatively unstable under the nonselective conditions of the environment [27].

Expressing biocontrol genes in other than a natural host for controlling plant pathogens was investigated by several researchers to provide stability and/or improved biological control rates. For instance, the *chiA* gene of *S. marcescens* was coexpressed with the *cry1Ac7* gene of *Bacillus thuringiensis* in *Pseudomonas fluorescens* to achieve more efficient biological control of *Eldana saccharina* larvae [6]. These kinds of studies generally use wild-type host because the natural colonizing behavior of the wild-type host is supposed to better exhibit the successful biocontrol activity. At least in the control of a soilborne pathogen of *F. oxysporum*, strain colonization was not necessary and *E. coli* might provide a useful host system for the development of recombinant biocontrol agents. To see the synergistic effect of more than 2 biocontrol mechanisms, however, it will be convenient to transfer the *chiA* gene into *P. fluorescens* 2112 strain producing pyoverdin [21] or *P. fluorescens* GL20 with siderophore activity [19]. These studies should require proper optimization of the inoculant bacteria.

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