

海洋細菌Alteromonas sp. Strain 79401株のキチン分解酵素遺伝子を導入した大腸菌による Botrytis属植物病原糸状菌の育成阻害

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In Vitro Growth Inhibition of Plant Pathogenic Fungi, *Botrytis* spp., by *Escherichia coli* Transformed with a Chitinolytic Enzyme Gene from a Marine Bacterium, *Alteromonas* sp. Strain 79401

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Abstract

Marine bacterium, *Alteromonas* sp. strain 79401 was shown to suppress the conidial growth of plant pathogenic fungi *Botrytis cinerea* and *B. fabae* *in vitro* by means of its high chitinolytic activity. To use the ability of this bacterium for biocontrol, a DNA fragment (*ca.* 12 kb) carrying the chitinolytic enzyme gene(s) was cloned into the plasmid pBR322. The resulting plasmid, pALCHII, was introduced into *Escherichia coli* strain DH5. *B. cinerea* and *B. fabae* could not grow in the vicinity of the transformed *E. coli* colonies *in vitro*. Conidial germination and hyphal growth were markedly suppressed. Swelling of the conidia and germ tubes and bursting of hyphal tips were observed under a microscope, suggesting that the chitinolytic enzyme affected the fungal cell walls. This chitinolytic enzyme gene might be useful for the biocontrol of *Botrytis* spp. and other plant pathogenic fungi.

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Key words: *Botrytis*, *Alteromonas*, chitinolytic enzyme, transformed *Escherichia coli*, biocontrol.

INTRODUCTION

Chitin, a polymer of *N*-acetylglucosamine, is a major component of the cell walls of many fungi, including plant pathogens³⁾, while higher plants do not contain chitin²⁵⁾. The addition of chitin to soil leads to an increase in the chitinolytic bacteria, especially actinomycetes, and fungi⁹⁾, which can attack the chitinous walls of soil fungi²⁰⁾ and reduce several soil-borne fungal diseases, *e.g.*, *Fusarium*^{19,33)} and *Rhizoctonia* diseases^{13,35)}.

The degradation of the cell walls of fungal pathogens by antagonistic microorganisms producing chitinolytic enzymes could be an effective method for biological control^{5,12,21,41)}. Furthermore, transferring chitinolytic enzyme genes to appropriate root- or leaf-colonizing bacteria could lead to novel biocontrol agents of plant

pathogenic fungi³⁸⁾.

Many bacteria, notably species within the genera *Serratia*, *Streptomyces* and *Vibrio*, produce extracellular chitinolytic enzymes^{7,23,26)}. Genes of these enzymes have been cloned from several terrestrial bacteria, *e.g.*, *S. marcescens*^{8,11,14,15,17,30,38)}, *S. liquefaciens*¹⁶⁾, *Str. plicatus*²⁹⁾ and *Str. lividans*²²⁾, *Bacillus circulans*⁴⁰⁾ and *Aeromonas hydrophila*⁶⁾.

Of the marine bacteria, chitinase and chitinase genes have been cloned from *Vibrio harveyi*^{36,37)}, *V. vulnificus*⁴²⁾ and *Alteromonas* sp.³⁹⁾ However, so far the marine bacteria chitinolytic enzyme genes have not been used for biocontrol agents of plant diseases. In this report, we describe the cloning of a chitinolytic enzyme gene from an isolate of *Alteromonas* sp. and its expression in *Escherichia coli*. Moreover, we demonstrate that the *E. coli* transformants expressing this chitinolytic activity were effective in suppressing the plant patho-

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genic fungi *Botrytis cinerea* and *B. fabae* *in vitro*.

MATERIALS AND METHODS

Bacterial and fungal strains, plasmids and culturing conditions. A highly chitinolytic bacterium was isolated from a seawater sample of the Pacific Ocean. It was gram-negative, flagellated, motile, aerobic and rod-shaped. The marine bacterium was identified as a member of genus *Alteromonas*⁴⁾ and designated as strain 79401.

The bacteria were grown on synthetic sea water (Jamarin S, Jamarin Lab.) agar medium containing 0.5% peptone (DIFCO), 0.1% yeast extract (DIFCO) (JSA-medium) and 0.1% (w/v) colloidal chitin. Colloidal chitin was prepared from crab shells (Sigma) by the HCl-method as described³¹⁾. The bacteria were also grown in the same medium without agar for isolation of chromosomal DNA. Cultures were incubated at 30°C.

The *E. coli* strain DH5 and the plasmid pBR322 were used; DH5 was cultured on Luria-Bertoni (LB) medium at 37°C as described¹⁸⁾. The plasmid pALCHII1 is a derivative of the plasmid pBR322, containing a *ca.* 12 kb *EcoRI* fragment which carried the chitinolytic enzyme gene of the *Alteromonas*. *E. coli* DH5 carrying pBR322 or pALCHII1 was grown in LB containing 100 µg/ml ampicillin (Sigma). For isolation and preservation of the transformed chitinolytic clones, LB agar (LBA) plates containing 0.1% colloidal chitin and 100 µg/ml ampicillin were used.

B. cinerea Persoon strain ICPR-1 was supplied from the Institute of Physical and Chemical Research (Wako-shi, Saitama, Japan), and *B. fabae* Sardina strain IFO 5895 was from the Institute for Fermentation (Osaka, Japan). To prepare the conidia, the fungi were grown on potato sucrose agar (PSA) plates (for *B. cinerea*) or on broad-bean sucrose agar (BSA) plates (for *B. fabae*) at 20°C for 5 days in the dark, followed by incubation under BLB light for 2 days and then again in the dark for 2 days. The conidia from both strains were stored at -40°C until use for germination assay.

Cloning of chitinolytic enzyme gene. *Alteromonas* chromosomal DNA was isolated by using a modification of the method described by Smith³²⁾, in which the bacterial cells were lysed by osmotic pressure of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) without any lysozyme and SDS, and the DNA then purified by equilibrium centrifugation in CsCl-ethidium bromide gradients¹⁸⁾. The *E. coli* DH5 competent cells were prepared by the method of Hanahan¹⁰⁾.

The plasmid vector pBR322 was cut with *EcoRI* (Toyobo) and treated with bacterial alkaline phosphatase (BAP, Toyobo). A partial *EcoRI* digest of *Alteromonas* chromosomal DNA was ligated into the vector at 16°C for 30 min using a DNA ligation kit (Takara). *E. coli* DH5 cells were transformed by the recombinant plasmids and selected on LBA plates containing 0.1% colloidal chitin and 100 µg/ml ampicil-

lin. The plates were incubated at 37°C for 24 hr and then at 30°C for 5 days. Colonies of *E. coli* DH5 containing plasmids with the chitinolytic enzyme gene(s) were chosen by the formation of a visible clearing zone around the colonies.

Southern hybridization. The *EcoRI* fragment of *ca.* 12 kb cloned into pBR322 was used as a probe. The fragment was labeled with photobiotin according to the manual of Photobiotin Labeling System (Bethesda Research Lab.). The genomic DNA from *Alteromonas* sp. strain 79401 digested with *EcoRI* was probed with the labeled fragment. Pre-hybridization, hybridization, washing, blocking, and detection procedures were performed according to the manual of BLUGENE Nonradioactive Nucleic Acid Detection System (Bethesda Research Lab.).

Chitinolytic enzyme assay. Chitinolytic activity of the culture plate exudates of *Alteromonas* sp. strain 79401 and *E. coli* DH5 transformed with pALCHII1 (pBR322 carrying *ca.* 12 kb fragment from the *Alteromonas*) or pBR322 (as a control) were assayed. The bacterium was streaked all over JSA (for *Alteromonas* sp.) or LBA (for *E. coli*) containing 0.1% colloidal chitin, and incubated at 30°C for 10 days. The culture plate of each bacterium was frozen at -30°C for 2 hr and then thawed at room temperature. The culture plate exudate was centrifuged at 10,000 rpm for 10 min, and the supernatant was passed through a sterilized nitrocellulose membrane filter (0.45 µm pore size, Advantec). The chitinolytic activity was assayed using *p*-nitrophenyl-β-D-N-acetylglucosaminide (pNP-NAG1, Sigma), *p*-nitrophenyl-β-D-N,N'-diacetylchitobioside (pNP-NAG2, Sigma) and *p*-nitrophenyl-β-D-N,N',N''-triacetylchitotriose (pNP-NAG3, Sigma) as substrates. The reaction mixtures containing 100 µl of the filtered exudate (2.5 µg crude protein for each) and 10 µg substrate in 100 µl MacIlvaine buffer (pH 6.0) were incubated at 37°C for 1 hr. The reaction was stopped by addition of 100 µl 1 M Na₂CO₃, and absorbance of released *p*-nitrophenol (pNP) at 405 nm was measured by a spectrophotometer.

Fungal germination assay. In order to make clearing zones visible around *Alteromonas* colonies, the bacteria were incubated on JSA plates containing 0.1% colloidal chitin at 30°C for 7 days. The transformed *E. coli* carrying a recombinant plasmid, pALCHII1, with the chitinolytic gene was grown on LBA plate containing 0.1% colloidal chitin and 100 µg/ml ampicillin at 37°C for 3 days, followed by incubation at 30°C for 12 days. As a control, the *E. coli* DH5 carrying pBR322 was grown on the same plate. The conidia prepared from *B. cinerea* or *B. fabae* were suspended in sterile distilled water at a concentration of 1×10⁴ conidia/ml, and 10 µl of the suspension was inoculated on the clearing zone or in its vicinity (within 5-10 mm), or in the vicinity of control *E. coli* colony. Germination of *Botrytis* conidia was calculated after 24 hr using a light microscope (3 replicates, 500 conidia each). Growth of the fungi was also followed with photomicrography.

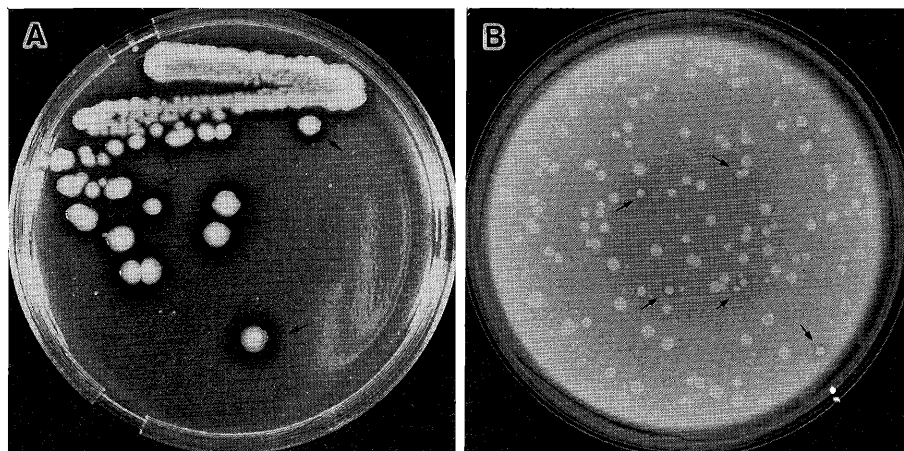


Fig. 1. Clearing zones formed on chitin plates by *Alteromonas* sp. strain 79401 (A) and *E. coli* DH5 transformed with pALCHII1 (B). The latter bacterium contains the chitinolytic gene(s) from the marine bacterium *Alteromonas* sp. within the plasmid pBR322. The chitinolytic transformants show up as rough colonies with an irregular margin and a narrow clearing zone. The strains were incubated as follows: A, at 30°C for 5 days on JSA-chitin medium; B, at 37°C for 2 days and at 30°C for 3 days on LBA-chitin medium.

RESULTS

Cloning of the chitinolytic enzyme gene

Chromosomal DNA prepared from *Alteromonas* sp. strain 79401 was partially digested with *Eco*RI, and ligated to *Eco*RI-cleaved and BAP-treated pBR322. The ligated mixture was used to transform *E. coli* DH5 competent cells. After 3 days of incubation, three of approximately 3800 transformants formed turbid clearing zones on LBA-chitin plates containing ampicillin, indicating chitin degradation. These clearing zones became distinct 5 days after plating (Fig. 1).

The recombinant plasmids extracted from the three chitinolytic transformants were analyzed by various restriction enzyme digestions. All three recombinant plasmids showed a ca. 12 kb DNA fragment upon diges-

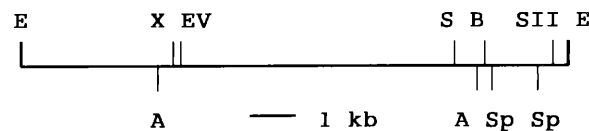


Fig. 2. Restriction map of the ca. 12 kb *Alteromonas* DNA insert of the plasmid pALCHII1. Abbreviations: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; S, *Sac*I; SII, *Sac*II; Sp, *Sph*I; X, *Xho*I.

tion with *Eco*RI (Fig. 2). Furthermore, all three clones had an identical restriction map, with two sites for *Ava*I and one site for *Bam*HI, *Eco*RV, *Sac*I and *Sac*II, suggesting that they carried the same gene.

In order to confirm that the *Eco*RI fragment of ca. 12 kb cloned into pBR322 was derived from the *Alteromonas* DNA, the fragment was labeled with photobiotin

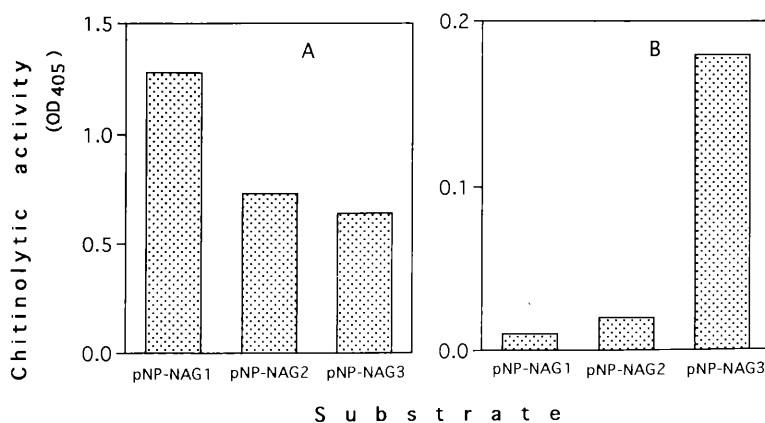


Fig. 3. Chitinolytic activities of the culture plate exudates of *Alteromonas* sp. strain 79401 (A) and *E. coli* transformant with pALCHII1 (B). The exudates used as enzyme solutions were prepared as in Materials and Methods. The activities of solutions with a crude protein concentration were measured by optical density (405 nm) of *p*-nitrophenol (pNP) released from pNP-NAG1, pNP-NAG2 and pNP-NAG3 after 1 hr of incubation at 37°C. The *E. coli* transformed with pBR322 showed no activities for any of the three substrates.

and used as a probe in Southern hybridization experiment. The probe hybridized only to a *ca.* 12 kb fragment in *Eco*RI digests of the *Alteromonas* chromosomal DNA (data not shown).

Both the culture plate exudates of the *Alteromonas* sp. strain 79401 and *E. coli* DH5 carrying the *ca.* 12 kb fragment from the *Alteromonas* in pBR322 showed chitinolytic activities (Fig. 3), while the exudate of pBR322 transformed DH5 showed no activities with any of three substrates. The exudate of the *Alteromonas* released pNP from pNP-NAG1 with highest activity, and from pNP-NAG2 and pNP-NAG3 with lower activities than from pNP-NAG1. On the other hand, the exudate of *E. coli* DH5 carrying the *ca.* 12 kb fragment released pNP from pNP-NAG3 with highest activity and from pNP-NAG2 with slight activity, but not from pNP-NAG1. These results indicate the presence of both exo-chitinase and endo-chitinase activities in the *Alteromonas* exudate, while only endo-chitinase activity was present in the transformed *E. coli* exudate. With pNP-NAG3, the activity per μ g crude protein of the *Alteromonas* exudate was three times higher than that of the transformed *E. coli*.

The plasmid carrying the chitinolytic gene was designated as pALCHI1, and the transformed *E. coli* was used for antifungal assays *in vitro*.

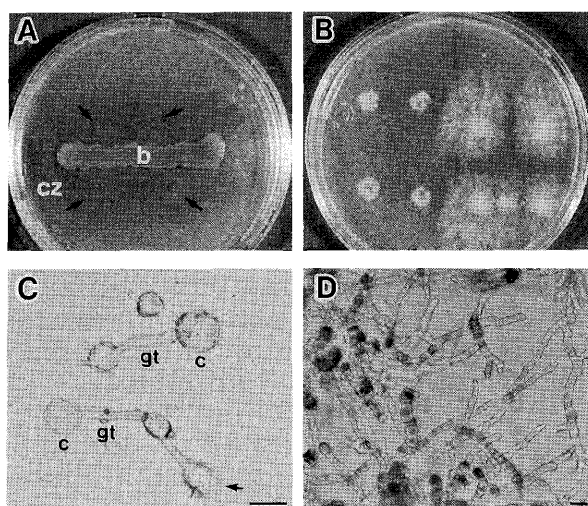


Fig. 4. Growth of *B. cinerea* on the clearing zone (cz) formed in JSA-chitin medium by *Alteromonas* sp. strain 79401 (A and C) and on the same medium without the bacterium (B and D). Ten μ l droplets of the fungal conidia (*ca.* 1×10^4 cfu/ml) were inoculated on the clearing zone formed around a 7 days old colony (b) of the bacterium (A: arrows) or on the medium without the bacterium (B), and the plates were incubated at 20°C for 10 days in the dark. Photomicrographs (C and D) show the swelling of conidia (c) and the germ tubes (gt) and bursting of the hyphal tips (arrow) on the clearing zone (C), but not on the control plate (D). Scales: 10 μ m.

Growth inhibition of *Botrytis* spp. by *Alteromonas* sp. and *E. coli* carrying pALCHI1

When the conidial suspension of *B. cinerea* was inoculated on and near the clearing zone produced by *Alteromonas* sp. on a JSA-chitin plate, the germination rate of the *B. cinerea* conidia was very low (0.1% on the clearing zone, 1.5% near it and 95.6% near the pBR322-transformed *E. coli* colony) so that formation of fungal colonies was almost completely inhibited. The corresponding rates of conidial germination for *B. fabae* were higher (44.1% on the clearing zone and 73.2% near it), but still significantly lower than in the control (100% near the pBR322-transformed *E. coli* colony). The conidia of both species became swollen and often burst within 24 hr of inoculation on the clearing zone. Under a light microscope, the cell walls appeared degraded, and swelling of the hyphal tips, cells with many septa and some chlamydospores were also observed as the surviving conidia germinated (Fig. 4). Thus the chitinolytic activity of *Alteromonas* seemed to be responsible for the inhibition of germination and growth in both *Botrytis* species.

Similarly to the results with *Alteromonas* on JSA-chitin plates, the pALCHI1 transformed chitinolytic *E. coli* DH5 on LBA-chitin plates with ampicillin inhibited almost completely the conidial germination of *B. cinerea* (germination rate 0.8%) within the clearing zone (Table 1). *B. fabae* was also affected markedly, although less than *B. cinerea*. Conidia inoculated further away from the bacterial colony, just outside the visible clearing zone, germinated slightly better (*B. cinerea*, 2.6%; *B. fabae*, 23.3%) than those on the clearing zone, but still clearly less than in the controls.

On the clearing zones of the pALCHI1-transformed *E. coli*, both *B. fabae* and especially *B. cinerea* showed an increased rate of conidial swelling (Table 1), swelling of hyphal tips, degradation of cell walls and other symptoms (Fig. 5) similar to those observed on the clearing zones produced by *Alteromonas*. The control *E. coli* transformed with plasmid pBR322 had no effect on the

Table 1. The incidence of conidial germination and swelling of *Botrytis cinerea* and *B. fabae* in the vicinity of transformed *Escherichia coli* DH5 expressing chitinolytic gene(s) from *Alteromonas* sp. strain 79401^{a)}

<i>E. coli</i> strain (plasmid)	<i>B. cinerea</i>		<i>B. fabae</i>	
	% germination	% swelling	% germination	% swelling
DH5 (pALCHI1)	0.8	27.3	18.2	7.5
DH5 (pBR322)	57.8	1.0	70.3	1.3
no bacteria	58.6	1.7	69.1	1.1

a) Data are averages of three replications, 500 conidia each. Antifungal assays on LBA-chitin plates are described in Materials and Methods.

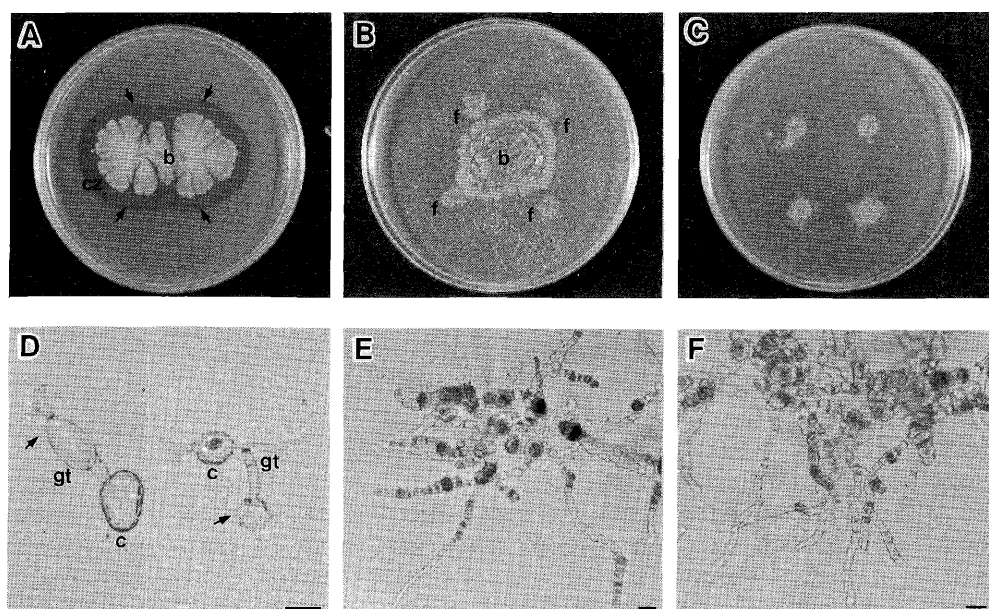


Fig. 5. Growth of *B. cinerea* on the clearing zone (cz) formed in LBA-chitin medium by chitinolytic *E. coli* DH5 transformed with pALCHI1 (A and D), around a colony of *E. coli* DH5 transformed with pBR322 (B and E) and on the medium without the bacteria (C and F). Ten μ l droplets of the fungal conidia (ca. 1×10^4 cfu/ml) were inoculated on the clearing zone formed around a 15 days old colony (b) of the pALCHI1 transformant (A : arrows), around a colony of the pBR322 transformant (B) or on the medium without bacteria (C), and the plates were incubated at 20°C for 10 days in the dark. Photomicrographs (D, E and F) show swelling of the conidia (c) and the germ tubes (gt) and bursting of the hyphal tips (arrow) on the clearing zone (D), but not in the controls (E and F). Scales : 10 μ m.

germination or swelling rates or on the morphology of *Botrytis* conidia. This indicates that the chitinolytic activity of *Alteromonas* was effectively transferred to *E. coli* within the 12 kb DNA fragment so as to suppress significantly the conidial germination and growth of *Botrytis* in our *in vitro* assay.

DISCUSSION

Only a few chitinolytic enzyme genes from marine bacteria have been cloned and expressed in *E. coli*, i.e., from *V. harveyi*³⁶⁾, *V. vulnificus*⁴²⁾ and *Alteromonas* sp.³⁹⁾ However, there have been no attempts to use these genes for the biocontrol of phytopathogenic fungi.

The marine bacterium *Alteromonas* sp. strain 79401 can cause clearing zones around colonies on chitin plates, indicating its ability to produce chitinolytic enzyme(s). When conidia of the fungi *B. cinerea* or *B. fabae* were inoculated on the clearing zone, conidial germination was suppressed significantly in both species. The conidia became swollen, with the appearance of spheroplasts; also the tips of germ tubes and hyphae swelled markedly. Although other antifungal substance(s) may be produced by *Alteromonas* sp., the morphological data suggested strongly that the chitinolytic activity of the bacterium was responsible for the inhibition of fungal colony formation, due to the degradation of the fungal cell walls and interference with cell wall synthesis. We have also observed that *Botrytis* conidia and hyphae are degraded in a similar

way on the chitin clearing zones produced by *S. marcescens*, as well as by the action of purified *Serratia* chitinase¹⁾.

S. marcescens is known to produce at least five different chitinolytic proteins⁸⁾. It was indicated that the *Alteromonas* sp. strain 79401 produces two types of chitinolytic enzymes (endo- and exo-types), but it is not yet known how many different enzymes the *Alteromonas* produces. Purification and characterization of the *Alteromonas* enzymes are in progress. It is known that exo-chitinase (β -N-acetylglucosamidase) can release N-acetylglucosamine from the non-reducing end of the chitin chain quite slowly and that endo-chitinases can cut the chitin oligomers longer than trimer quite rapidly. Therefore, the enzyme secreted by the pALCHI1-transformed *E. coli* might be an endo-chitinase, not an exo-chitinase. The endo-chitinase activity shown by the pALCHI1-transformed *E. coli* was three times lower than that by the *Alteromonas* at the same crude protein level. There should be some difficulties for the *Alteromonas* gene to be expressed in *E. coli*, especially in a 12 kb long fragment. It is also thought that endo- and exo-chitinase of the *Alteromonas* worked together to show higher activity than the transformed *E. coli*. We are now subcloning to elucidate the size and regulation of the chitinolytic enzyme gene in our clone. In order to use all the chitinolytic activity of the *Alteromonas* sp. strain 79401 for biocontrol of plant pathogenic fungi, we are also trying to clone other chitinolytic enzyme gene(s) from the strain.

Since the *Alteromonas* is a marine bacterium, it needs a high concentration of NaCl for growth. Therefore, its direct use as a biocontrol agent against plant pathogenic fungi seems not feasible. As a first step toward developing alternative transformed biocontrol agents, we have cloned into *E. coli* a 12 kb DNA fragment containing the *Alteromonas* gene responsible for the chitinolytic activity. The gene was found to be expressed well in *E. coli* and the transformed *E. coli* inhibited *Botrytis* growth effectively *in vitro* and in a manner similar to that of *Alteromonas*.

Apart from the marine *Alteromonas*, several other, terrestrial bacteria producing chitinolytic enzymes have been shown to suppress plant pathogenic fungi, e.g., *Arthrobacter* sp.^{2, 24)}, *Serratia* sp. and *Hafnia* sp.³⁴⁾ inhibit *Fusarium* spp. *Serratia* sp. also suppresses *Sclerotium* fungi^{27, 28)}. However, to our knowledge there are no previous demonstrations of bacterial chitinolysis affecting *Botrytis* fungi, except by *S. marcescens* as recently found by us¹⁾. We have shown here again that *Botrytis* spp. are susceptible to bacterial chitinolytic enzymes, even when the enzyme originates from a marine bacterium and is expressed in a heterologous host. This indicates a potential of biological control by transformed chitinolytic antagonists against *Botrytis* fungi.

Previously, *Serratia* chitinase has been expressed in *Pseudomonas* sp. isolates, known to be good plant root colonizers, for biocontrol of *F. oxysporum*³⁸⁾. The *Serratia* chitinase has been expressed also in *E. coli* for biocontrol of *Sclerotium*³⁰⁾. We intend to transfer the *Alteromonas* chitinolytic enzyme gene into root- or leaf-colonizing bacteria to suppress plant pathogenic fungi.

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和 文 摘 要

平八重一之・平田暁子・阿久津克己・原 三郎・Ilkka HAVUKKALA・西沢洋子・日比忠明：海洋細菌 *Alteromonas* sp. 79401 株のキチン分解酵素遺伝子を導入した大腸菌による *Botrytis* 属植物病原糸状菌の育成阻害

海洋細菌 *Alteromonas* sp. 79401 株は、その高いキチン分解活性によって、培地上で植物病原菌 *Botrytis cinerea* および *B. fabae* の分生胞子の生育を阻害した。この海洋細菌のキチン分解活性をバイオコントロールに利用する目的で、本菌株のキチン分解酵素遺伝子を含む約 12 kb の染色体 DNA 断片をプラスミド pBR322 を用いてクローニングした。得られた組換えプラスミド pALCHII を導入した大腸菌 DH5 株を *B. cinerea* あるいは *B. fabae* と対峙培養したところ、形質転換株のコロニーの周囲で培地中のコロイダルキチンが分解されるとともに、両病原菌の著しい生育阻害が認められた。すなわち、両菌の胞子発芽および菌糸の生育が阻害され、胞子と発芽管の膨潤および菌糸先端部の分解が光学顕微鏡下で観察された。このことは、導入した遺伝子が大腸菌で発現し、菌体外に分泌されたキチン分解酵素によって両菌の細胞壁が分解されたことを示している。以上の結果から、海洋細菌 *Alteromonas* sp. 79401 株のキチン分解酵素遺伝子を *Botrytis* 属菌およびその他の植物病原菌類のバイオコントロールに利用し得る可能性が示唆された。