High Level Expression of *Fusarium* Alkaline Protease Gene in *Acremonium chrysogenum*

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We transformed *Acremonium chrysogenum* with the genomic DNA of the alkaline protease (Alp) from *Fusarium* sp. S-19-5 including its promoter. Most of the transformants thus obtained produced a large amount of Alp. PCR and Southern hybridization analysis of genomic DNAs from these transformants showed chromosomal integration of the full-length Alp gene. SDS-PAGE analysis of the supernatant from the transformants showed the presence of *Fusarium* Alp. The amino terminus of the Alp produced in *A. chrysogenum* was identical to that of native *Fusarium* Alp. These results indicate that the Alp promoter, signal sequence, and introns functioned correctly in *A. chrysogenum*. One of the transformants produced more than 4 mg of the Alp per liter in a jar fermentor.

*Acremonium chrysogenum* has been used for the industrial production of the beta-lactam antibiotic cephalosporin C for a number of years. It is therefore considered to be quite safe as a recombinant host like *Aspergillus* and *Trichoderma*. In 1985, Queener et al. developed a transformation system for *Acremonium chrysogenum* using the *Escherichia coli* hygromycin B phosphotransferase gene as a dominant marker.1) Using this transformation system, Isogai et al. created a 7-aminocephalosporanic acid producing *A. chrysogenum* by inserting the genes encoding L-amino acid oxidase from *Fusarium solani* and cephalosporin acylase from *Pseudomonas diminuta*.2)

However, *A. chrysogenum* has not yet been used successfully as a recombinant host for the secretion of heterologous proteins. This is because strong promoters and suitable signal sequences that can function in *A. chrysogenum* have not yet been found.

We have cloned the alkaline protease (Alp) gene of *Fusarium* sp. S-19-5 and identified the promoter region.3) As *Fusarium* is known to be taxonomically close to *A. chrysogenum*,4,5) we attempted to use the *Fusarium* Alp promoter and signal sequence for the production of heterologous proteins by *A. chrysogenum*.

In this paper, we report the efficient secretion of *Fusarium* Alp by *A. chrysogenum* using its own promoter and signal sequence.

Materials and Methods

**Microorganisms and plasmids.** *E. coli* DH-1*<sup>6</sup> and JM109*<sup>7</sup> were used as the recombinant hosts. *A. chrysogenum* ATCC11550 was used as the host for the expression of the Alp gene. Plasmids pUC118 and pUC119 were purchased from Takara Shuzo Co. Plasmid pGH21 was constructed from pGH2.8)

**Media and culture conditions.** *E. coli* was grown in LB medium.9) when necessary 50 µg/ml of ampicillin was added. Plate medium for detection of Alp activity contained malt extract, 20 g/liter; galactose, 20 g/liter; polypeptone, 1 g/liter; casein, 10 g/liter; and agar, 20 g/liter, and was adjusted to pH 6.8. Liquid medium for seed and main culture contained sucrose, 30 g/liter; L-methionine, 5 g/liter; soy bean flour, 32 g/liter; and CaCO₃, 1.5 g/liter, and was adjusted to pH 6.8. Trypticase Soy Agar (40 g/liter) (Becton Dickinson) was used for slant medium. The recombinant microorganisms were grown at 28°C for 3 days in the seed medium. Samples (3 ml) of the culture were inoculated into 50 ml of the main medium in a 200-ml Erlenmeyer flask and incubated at 28°C for 5 days on a rotary shaker. Culture in a 2-liter jar fermentor was incubated at 28°C for 10 days using 1 liter of the main medium.

**Transformation.** *E. coli* was transformed by the CaCl₂/ RpcI procedure.10) *A. chrysogenum* ATCC11550 was transformed by the method of Queener et al.11)

**DNA isolation.** Total DNAs of *A. chrysogenum* ATCC11550 and the transformants were prepared as described by Rueder and Broda.10) Plasmid DNA was isolated by the alkaline extraction procedure.10)

**PCR.** Two oligonucleotide primers, 5’-CAAAACTCACTTCTCAACC-3’ (the 5’-non coding region of the Alp gene from *Fusarium* sp. S-19-5, -38 to -19) and 5’-CATCTCTCATTCCTCCAG-3’ (the 3’-non coding region of the Alp gene, +1337 to +1355),12) were synthesized using a DNA synthesizer (Applied Biosystems; Model 381A). The Alp DNA was amplified by the polymerase chain reaction (PCR)13) using the two oligonucleotides as primers, genomic DNAs extracted from *A. chrysogenum* ATCC11550 and the transformants as templates, a Gene Amp DNA amplification reagent kit, and a DNA Thermal Cycler (Perkin Elmer Cetus).

**Southern hybridization.** Genomic DNAs extracted from *A. chrysogenum* ATCC11550 and its transformants were digested with EcoRI, electrophoresed on 1% agarose gel, and transferred to nylon membrane filter by the method of Southern.14) A DNA fragment amplified by PCR was labelled with an ECL gene detection system (Amerham) and used as a probe. Hybridization was done as described by Manuatis et al.7)

**Enzyme activity assay.** Protease activity was measured by the method of Tomoda et al.16) One unit of protease activity was defined as the amount that catalyzed the release of 1 µg of tyrosine per minute. Specific activity of purified Alp was taken as 5800 units/mg.

**Amino terminal sequencing.** The *Fusarium* Alp protein secreted into the culture supernatant of an *A. chrysogenum* transformant was separated by SDS polyacrylamide gel electrophoresis and transferred to a PVDF Transfer Membrane (Applied Biosystems). The adsorbed protein was then sequenced using a gas-liquid solid phase sequenator (Applied Biosystems: Model 470A).

Results

**Secretion of the Alp protein of *Fusarium* sp. S-19-5 by *A. chrysogenum*.**

The genus *Fusarium* is not suitable for use as a recombinant host because it is considered a plant pathogen.
A 2.8-Kbp fragment obtained by digestion of M13-Alp with BamHI was subcloned into pUC119. A 3.0-Kbp fragment including the A. chrysogenum GAPDH promoter and hygromycin B phosphotransferase gene was also obtained by the digestion of pGH21 with HindIII. This fragment was inserted into the HindIII site of the plasmid described above to form plasmid, pNH1.

**Table**

<table>
<thead>
<tr>
<th>Production of <em>Fusarium</em> Alkaline Protease in *Acremonium chrysogenum/pNH1</th>
<th>Dry cell weight (g/liter)</th>
<th>Activity (units/ml)</th>
<th>Production of alkaline protease (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. chrysogenum ATCC11550 Transformant 1</td>
<td>34</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Transformant 2</td>
<td>48</td>
<td>104</td>
<td>0</td>
</tr>
<tr>
<td>Transformant 3</td>
<td>33</td>
<td>8460</td>
<td>1460</td>
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<tr>
<td>Transformant 4</td>
<td>78</td>
<td>6980</td>
<td>1200</td>
</tr>
</tbody>
</table>

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Fig. 3. Southern Blot Analysis of the *Fusarium* Alp Gene in *A. chrysogenum* Transformants.

Ten μg of genomic DNAs from *A. chrysogenum* ATCC11550 (lane 1) and *A. chrysogenum* transformants (lanes 2-5) was digested with EcoRI, electrophoresed on a 1% agarose gel and transferred onto a nylon membrane. Hybridization was done using a 1.4-Kbp Alp DNA fragment amplified by PCR as a probe.

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**Fig. 2.** Secretion of *Fusarium* Alp by *A. chrysogenum* Transformants. *A. chrysogenum* ATCC11550 (a) and *A. chrysogenum* pNH1 (b) were grown on slant medium, and the spores were suspended in distilled water. After appropriate dilution, spores were spread on casein plates and cultivated at 25°C for 6 days.

We, therefore, chose *A. chrysogenum* which is taxonomically close to *Fusarium*.

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**Fig. 1.** Construction of the Expression Plasmid for *Fusarium* Alp, pNH1.

A 2.8-Kbp fragment obtained by digestion of M13-Alp with BamHI was subcloned into pUC119. A 3.0-Kbp fragment including the *A. chrysogenum* GAPDH promoter and hygromycin B phosphotransferase gene was also obtained by the digestion of pGH21 with HindIII. This fragment was inserted into the HindIII site of the plasmid described above to form plasmid, pNH1.

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We, therefore, chose *A. chrysogenum* which is taxonomically close to *Fusarium*. To find whether *A. chrysogenum* can secrete heterologous proteins using the *Fusarium* Alp promoter and signal sequence, we constructed a plasmid, pNH1, for the expression of the Alp gene (Fig. 1). Plasmid pNH1 is an insertion type plasmid and contains the *Fusarium* Alp promoter and signal sequence for secretion of the Alp itself and the hygromycin B phosphotransferase (HPT) gene downstream from the *A. chrysogenum* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter as a selective marker. This plasmid was introduced into *A. chrysogenum* ATCC11550, and seven transformants were obtained. Appropriate dilutions of spore suspension from the transformants and original strain were plated on casein plates to find whether the transformants secreted *Fusarium* Alp. When the plates were incubated for 6 days at 28°C, clotting was observed around most of the transformants (Fig. 2b) but not around the original strain (Fig. 2a) or a few of the transformants. We selected two transformants (Transformant 1, 2), around which clotting was not observed, and two transformants (Transformants 3, 4), around which clotting was observed, and cultured them in liquid medium. The two clot-inducing transformants showed forty-fold higher protease activity than the original strain, while the protease activities of the two non-clot-inducing transformants were less than that of the original strain (Table).

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**Analysis of the Alp gene of Fusarium sp. S-19-5 inserted in A. chrysogenum**

To examine whether the Alp gene had been inserted into
the genomic DNAs of the transformants. Southern blot analysis was done using EcoRI digested genomic DNAs extracted from the original strain and the transformants. We prepared a probe of a 1.4-Kbp Alp DNA fragment by PCR using oligonucleotides coding for 20 bp of the 5'-non coding region (−19 to −38) and 19 bp of the 3'-non coding region (+1337 to +1355) of the Alp gene as primers and pNH1 as a template. No band was detected from the sample of the original strain and a non clot-inducing transformant (Fig. 3, lanes 1, 3), but a 9-Kb band that corresponded to the size of pNH1 was detected from the sample of transformant 3 (Fig. 3, lane 4). On the other hand, several bands were detected from the sample of transformant 4 (Fig. 3, lane 5). When comparing these two transformants, the Alp gene might be tandemly inserted in the genomic DNA of transformant 3 and rearranged in several sites in the genomic DNA of transformant 4 after integration. Some bands were also detected with the sample of transformant 1 though it did not show Alp activity (Fig. 3, lane 2). To examine whether each transformant contained the full length of the Alp gene or not, PCR analysis was next done using genomic DNAs as templates. When the reaction mixtures were electrophoresed on an agarose gel, a 1.4-Kbp band that coincided with the deduced length of the amplified Alp DNA appeared from the two clot-inducing transformants (Transformants 3, 4; Fig. 4, lanes 5, 6), while no band appeared with those from the original strain (Fig. 4, lane 2) or the two non-clot-inducing transformants (Transformants 1, 2; Fig. 4, lanes 3, 4). On the other hand, when PCR was done using the oligonucleotides coding for the 5'- and the 3'-terminus of the HPT gene as primers, the HPT gene was amplified from the genomic DNAs of all the transformants but not detected from that of the original strain (data not shown). From this result, the genomic DNA of transformant 1 was not thought to contain the expression unit of Alp.

Characterization of the secreted Alp protein

To confirm that the secreted proteases were identical to Fusarium Alp, SDS-polyacrylamide gel electrophoresis of the supernatant from the original strain (Fig. 5, lane 2) and transformant 3 (Fig. 5, lane 3) was carried out. A major band of molecular weight 32,500, the mobility of which was identical to that of purified native Alp (Fig. 5, lane 1), and many minor bands were detected with the supernatant of the transformant but not that of the original strain. The amino terminal amino acid sequence of the major band was Ala-Ile-Thr-Gln-Gln-Gly-Gly-Ala, the same as the eight amino terminal residues of the native Fusarium Alp.31 The minor bands were deduced to be partial fragments of the Alp generated by autoproteolysis since the bands were also observed with the purified native Alp. Thus, Fusarium Alp was confirmed to be secreted as a mature protein from the A. chrysogenum transformant bearing a plasmid designed to express prepro-Alp in the cell.

Production of Fusarium Alp in a jar fermentor

When A. chrysogenum transformant 3 was cultured in a 2-liter jar fermentor, the amount of Alp secretion increa-

![Fig. 4. PCR Analysis of the Fusarium Alp Gene in A. chrysogenum Transformants.](image)

PCR was done using 1 μg of genomic DNAs from A. chrysogenum ATCC11550 (lane 2) and A. chrysogenum transformants (lanes 3-6) as templates. The reaction mixtures (10 μl) were electrophoresed on a 1% agarose gel. Lambda DNA digested with EcoRI was used as size markers (lane 1).

![Fig. 5. SDS PAGE Analysis of Fusarium Alp Secreted by A. chrysogenum Transformant.](image)

A. chrysogenum ATCC11550 (lane 2) and A. chrysogenum/pNH1 (lane 3) were inoculated into 50 ml of the liquid medium in a 200-ml Erlemeyer flask and incubated at 28°C for 5 days. A sample (10 μl) of the culture supernatant and 4 μg of purified enzyme (lane 1) were electrophoresed on an SDS polyacrylamide gel. Molecular markers are phosphorylase b (M = 97,000), bovine serum albumin (66,000), ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (22,000), and hen egg lysozyme (14,000) (lane M).
ed in proportion to the cell growth (Fig. 6). Finally, 4.2 g/liter of Alp, calculated on the basis of the specific activity of purified Alp from *Fusarium* sp. S-19-5, had accumulated in the culture medium after cultivation for 240 h.

**Discussion**

There has been an increasing number of reports on heterologous protein secretion using filamentous fungi such as *Aspergillus nidulans*, A. *niger*, A. *oryzae*, and *Trichoderma reesei*, and the promoters and signal sequences originate from *Aspergillus* or *Trichoderma*. We succeeded here in the accumulation of over 4 g/liter of *Fusarium* Alp in the culture medium of *Acremonium chrysogenum* using its promoter and signal sequence. This is the first report of heterologous protein secretion from filamentous fungi using a heterologous promoter and signal sequence, though heterologous selective markers containing their promoter, such as *amS*, *argB*, and *nidd*, are known to be able to transform filamentous fungi. Furthermore, the expression level obtained here (4.2 g/liter) is greater than that for *Rhizomucor miehei* acidic protease (3.3 g/liter) in *Aspergillus oryzae* and the highest level that we know for a heterologous expression system. We believe that *A. chrysogenum* has the potential to be a general host for secretion of heterologous proteins in addition to the currently used *Aspergillus* and *Trichoderma*. We also expect that this finding may be a useful example for heterologous protein secretion using a heterologous strong promoter and suitable signal sequence in other safe recombinant hosts.

SDS-PAGE analysis (Fig. 5) showed that the molecular weight of the Alp from the supernatant of the transformant was identical to that of the purified native Alp, which suggests that the three introns from the *Fusarium* Alp gene are removed in *A. chrysogenum*. As the *Fusarium* Alp gene has the consensus sequence of introns of filamentous fungi, *A. chrysogenum* could process *Fusarium* introns. The amino terminal amino acid sequence of the Alp secreted by the *A. chrysogenum* transformant was also identical to that of the native Alp, which suggests a common mechanism is present for the recognition of the pre and pro sequences in *Fusarium* and *Acremonium*.

When *A. niger* transformants were obtained by introducing pNH1 into *A. niger*, Alp was not secreted into the culture medium (data not shown), though the gene was detected by PCR analysis. Therefore, the combination of *A. chrysogenum* host and the expression vector having the *Fusarium* Alp promoter and signal sequence is important to achieve an effective secretion system. Attempts to develop a new production system for useful proteins using this system are now in progress.

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**References**