Family 19 chitinases of *Streptomyces* species: characterization and distribution

Takeshi Watanabe,¹ Ryo Kanai,¹ Tomokazu Kawase,¹ Toshiaki Tanabe,² Masaru Mitsutomi,² Shohei Sakuda³ and Kiyotaka Miyashita⁴

Author for correspondence: Takeshi Watanabe. Tel: +81 25 262 6647. Fax: +81 25 262 6854. e-mail: wata@agr.niigata-u.ac.jp

Chitinase C from *Streptomyces griseus* HUT6037, described in 1997, is the first family 19 chitinase found in an organism other than higher plants. In this study, some properties of chitinase C were compared with those of family 18 bacterial chitinases, and the distribution of family 19 chitinases in *Streptomyces* species was investigated. The specific hydrolysing activity of chitinase C against soluble and insoluble chitinous substrates was markedly higher than those of bacterial family 18 chitinases. Chitinase C exhibited marked antifungal activity, whereas the other bacterial chitinases examined had no antifungal activity. Chitinase C was insensitive to allosamidin, whereas the family 18 bacterial chitinases were sensitive. Taking advantage of this insensitivity to allosamidin, a search was made for family 19 chitinases in various *Streptomyces* species. Chitinases insensitive to allosamidin were detected in the culture supernatants of all tested *Streptomyces* species. Southern hybridization analysis using a labelled DNA fragment corresponding to the catalytic domain of chitinase C strongly suggested that these species have genes similar to the *chiC* gene of *S. griseus* HUT6037. DNA fragments corresponding to the major part of the catalytic domains were amplified by PCR. The amplified fragments encoded amino acid sequences very similar to that of the corresponding region of chitinase C. Therefore, it was concluded that *Streptomyces* species generally possess family 19 chitinases which are very similar to chitinase C. Comparison of their amino acid sequences with those of plant family 19 chitinases revealed that *Streptomyces* family 19 chitinases are class IV type in terms of the presence and positions of deletions of amino acid sequences which are characteristic of plant class IV chitinases.

Keywords: chitinase, *Streptomyces*, allosamidin

INTRODUCTION

Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyse the degradation of chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine. Chitin, one of the most abundant natural polymers, is found in the cuticles of insects, the shells of crustaceans, and the cell walls of many fungi. Chitinases are present in a wide range of organisms, including organisms that do not contain chitin, such as bacteria, viruses, higher plants and animals, and play important physiological and ecological roles. Chitinases so far sequenced are classified into two different families, families 18 and 19, in the classification system of glycosyl hydrolases, based on the amino acid sequence similarity of their catalytic domains (Henrissat, 1991; Henrissat & Bairoch, 1993; Davies & Henrissat, 1995). Family 18 contains chitinases from bacteria, fungi, viruses and animals, and some plant chitinases (classes III and V). Family 19 contains plant classes I, II and IV chitinases and the recently identified *Streptomyces griseus* chitinase C (Ohno et al., 1996). The chitinases of the two families do not share amino acid sequence similarity, and they have completely different three-dimensional (3D) structures; they are therefore thought to have different evolutionary origins. The catalytic domains of family 18 chitinases have an α/β8-barrel fold as demonstrated by 3D-structural analyses of hevamine (Terwisscha van Scheltinga et al., 1994) and *Serratia marcescens* chitinase A (Perrakis et
In addition to the difference in 3D structure, chitinases of the two families show several important differences in their biochemical properties. For example, family 18 chitinases hydrolyse the glycosidic bond with retention of the anomic configuration (Armand et al., 1994; Iseli et al., 1996), whereas family 19 chitinases hydrolyse with inversion (Ohno et al., 1996; Fukamizo et al., 1995). Family 18 chitinases are sensitive to allosamidin, but a family 19 chitinase from higher plants has been shown to be insensitive (Koga et al., 1996). Family 18 chitinases hydrolyse GlcNAc-GlcNAc and GlcNAc-GlCN linkages, whereas family 19 chitinases hydrolyse GlcNAc-GlCN linkages and GlcN-GlCN (Ohno et al., 1996; Mitsutomi et al., 1996, 1997). These differences are probably common between all members of the two families and arise from the differences in their catalytic mechanisms. Substrate-assisted catalysis is the most widely accepted model for the catalytic mechanism of family 18 chitinases (Tews et al., 1997; Brameld et al., 1998), whereas a general acid-base mechanism has been suggested to be the catalytic mechanism for family 19 chitinases (Hart et al., 1995; Garcia-Casado et al., 1998).

Chitinase C from S. griseus HUT6037 is the first family 19 chitinase found in an organism other than higher plants (Ohno et al., 1996). It has a catalytic domain homologous to those of plant class I, II and IV chitinases, and an N-terminal chitin-binding domain. Plant chitinases in classes I and IV have a cysteine-rich domain (also referred to as the wheatgerm agglutinin domain) at their N-termini which is involved in chitin binding (Iseli et al., 1993; Raikhel et al., 1993; Shinshi et al., 1990). Class IV chitinases are smaller than class I chitinases due to deletions in both the cysteine-rich domain and the catalytic domain (Collinge et al., 1993). Class II chitinases are homologous to those of class I and IV but lack the cysteine-rich chitin-binding domain. The chitin-binding domains of plant chitinases and S. griseus chitinase C do not share significant sequence similarity. The chitin-binding domain of S. griseus chitinase C has obvious sequence similarity to the chitin-binding domains and probable chitin-binding domains of some bacterial family 18 chitinases, including chitinases A1 and D1 of Bacillus circulans WL-12 (Ohno et al., 1996).

In the present study, we constructed an expression system for chitinase C in Escherichia coli for further characterization, and found some important characteristics of this chitinase. In addition, we demonstrated the general occurrence of family 19 chitinases in Streptomyces.

METHODS

Strains, plasmids, media and culture conditions. E. coli BL21(DE3) was used for the expression of the chiC gene of S. griseus HUT6037. E. coli JM109 was used as a general cloning host. Streptomyces species used in this study are summarized in Table 1. Recombinant plasmid pGC01, carrying a 1.7 kb DNA insert from S. griseus HUT6037 containing the chiC gene encoding chitinase C, was described previously (Ohno et al., 1996). The regions upstream and downstream of the coding sequence of chiC were removed from the 1.7 kb DNA insert of pGC01 by Xbal and BamHI digestion, the resulting DNA fragment (approx. 1.0 kb) was ligated with Xbal- and BamHI-digested pUC119, and the resultant plasmid was designated pGC01A. E. coli cells carrying the recombinant plasmids were grown on Luria–Bertani (LB) medium supplemented with 100 µg ampicillin ml⁻¹. Streptomyces cells were grown at 30 °C in a medium containing 0.2% colloidal chitin, 0.05% KCl, 0.1% K2HPO4, 0.05% MgSO4.7H2O (pH 7.0) and 0.001% FeSO4 for enzyme production, and 10% mannitol, 0.2% peptone, 0.1% meat extract and 0.1% yeast extract (pH 7.0) for chromosomal DNA preparation.

Construction of expression plasmid pGC02. To get higher expression of the chiC gene, plasmid pGC02 was constructed using pET12a as the vector plasmid. The coding region of chiC was amplified by PCR and amplified fragment was ligated with Ndel- and BamHI-digested pET12a. The forward primer for PCR (5’-GATCATATGATCGAGTGTCA-TC3’) is identical to the N-terminal region of the chiC gene except that the initiation codon was changed from GTG to ATG and an additional sequence consisting of an Ndel cleavage site was added at its 5’ end. The reverse primer (5’-GGCGGATCAGATCAGCTCAG-3’) is complementary to the C-terminal region of the chiC gene and contained a BamHI cleavage site.

Expression of chiC and purification of its product. E. coli BL21(DE3) cells harbouring pGC02 were grown at 30 °C on LB medium containing 100 µg ampicillin ml⁻¹. When cells reached an OD600 of 0.7, IPTG (final concentration 0.4 mM) was added and the cells were cultivated for another 5 h at 30 °C. After collecting the cells by centrifugation, chitinase C secreted into the periplasmic fraction was extracted by the cold osmotic shock procedure as described by Manoil & Beckwith (1986). The extracted periplasmic proteins containing chitinase C were collected by ammonium sulfate precipitation (60% saturation), dissolved in 20 mM phosphate buffer (pH 6.0), dialysed against 2 mM sodium phosphate buffer (pH 6.0) and lyophilized. Lyophilized proteins were dissolved in a small volume of 20 mM phosphate buffer (pH 6.0) and applied onto a hydroxylapatite column (3.5 × 13 cm) previously equilibrated with the same buffer. Proteins were eluted with the same buffer and the peak fractions containing chitinase C were collected, dialysed against 2 mM sodium phosphate buffer (pH 6.0), and lyophilized.

Purification of other chitinases. Serratia marcescens chitinases A and C1 (Suzuki et al., 1998, 1999), and B. circulans chitinases A1 and D1 (Armand et al., 1994), were purified as described previously.

B. circulans chitinase C1 was produced by E. coli JM109 harbouring the recombinant plasmid pALC11 (Alam et al., 1995) and extracted from the periplasmic space of the cells by the cold osmotic shock procedure. Proteins in the periplasmic fraction were collected by ammonium sulfate precipitation (60% saturation) and applied to a hydroxylapatite column (1.5 × 15 cm) washed with 1 mM phosphate buffer (pH 6.0). Elution was done with a gradient of 1–400 mM sodium phosphate buffer (pH 6.0) with a flow rate of 25 ml min⁻¹. The fractions containing chitinase C1 were pooled, concentrated and further purified by HPLC with a Shim-pack PA-DEAE column in a liquid chromatograph (LC-6A system, Shimadzu). Elution was performed with 20 mM Tris/HCl buffer (pH 7.5) with a 0–5 M NaCl gradient.
Serratia marcescens chitinase B was produced by E. coli DH5α cells harbouring plasmid pMCB7 carrying the cloned chiB gene (Watanabe et al., 1997). The cells were grown for 24 h in LB medium supplemented with 100 µg ampicillin ml⁻¹ and 0.4 mM IPTG, collected by centrifugation and disrupted by sonication. After removing unbroken cells and debris, proteins were collected by ammonium sulfate precipitation (20–40% saturation). The precipitate was dissolved in a small volume of 1 mM sodium phosphate buffer (pH 6.0) and applied to a hydroxyapatite column (3×7 cm) previously equilibrated with the same buffer, and eluted with the same buffer. The unadsorbed protein fractions containing chitinase B were collected and lyophilized. Lyophilized chitinase B was dissolved in a small volume of 1 mM phosphate buffer (pH 6.0) and further purified by chitin affinity column chromatography as described previously (Suzuki et al., 1998).

**Antifungal activity.** A suspension of conidia of Trichoderma reesei was adjusted to 2.5–5×10⁵ ml⁻¹ and a paper disk placed on the centre of a potato dextrose agar plate was soaked with 40 µl of the suspension. After 24 h incubation at 30 °C, blank paper disks were placed around the T. reesei colony and solutions of various chitinases were added to these disks. The plates were incubated for approximately 12 h and inhibition of hyphal extension was evaluated by visual inspection.

**Genomic DNA extraction and Southern hybridization.** Chromosomal DNAs of various Streptomyces species were extracted from the mycelia by the method described by Hopwood et al. (1985) with minor modifications. For Southern hybridization, restriction-enzyme-digested DNA was fractionated in a 0.7% agarose gel, transferred onto a nylon membrane (Hybond-N, Amersham) by the capillary method, and hybridized with a ³²P-labelled chiB probe which included the entire catalytic domain of chitinase B.

**PCR and nucleotide sequence determination of family 19 chitinases of Streptomyces species.** A portion of the genes encoding family 19 chitinases of various Streptomyces species was amplified by PCR. Primers for PCR were designed based on the regions of conserved amino acid sequences in the catalytic domains of chitinase C from S. griseus HUT6037 and plant family 19 chitinases. Forward and reverse primers corresponded to the amino acid sequences from Lys-134 to Ala-142 and from Ile-256 to Cys-262 of chitinase C, respectively. Amplified fragments were ligated with T-vector p17Blue (Novagen) and maintained in E. coli JM109. Nucleotide sequences of amplified fragments in the T-vector were determined with an automated laser fluorescence sequencer (model 4000L, LI-COR). Sequencing reactions were done by using a Thermosequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) according to the supplier’s instructions with double-stranded templates. Nucleotide sequence data were analysed using the GENETYX system (Software Kaihatsu Co.).

**SDS-PAGE.** SDS-PAGE in slabs was conducted as described by Ames (1974), using the buffer system of Laemmli (1970). When necessary, renaturation of enzymes in polyacrylamide gels and detection of chitinase activity was performed as described previously (Watanabe et al., 1990).

**Enzyme and protein assay.** Chitinase activity was measured by a modification of Schales’ procedure (Imoto & Yagishita, 1971) with either colloidal chitin or soluble chitin as the assay substrate. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol reducing sugar min⁻¹. Protein concentration was measured by the Lowry method, using bovine serum albumin as the standard.

**Chemicals.** Glycol chitin and colloidal chitin were prepared from powdered crab shell chitin purchased from Funakoshi.

<table>
<thead>
<tr>
<th>Table 1. Strains used in this study</th>
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<tr>
<td><strong>Organism</strong></td>
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<tr>
<td>Streptomyces lividans 66</td>
</tr>
<tr>
<td>Streptomyces coelicolor A3(2)</td>
</tr>
<tr>
<td>Streptomyces lividans ISP5434</td>
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<tr>
<td>Streptomyces coelicercis ISP5421</td>
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<tr>
<td>Streptomyces cyanoolor ISP5241</td>
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<tr>
<td>Streptomyces eurythermus ISP5014</td>
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<tr>
<td>Streptomyces flavovirens ISP150</td>
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<tr>
<td>Streptomyces griseus HUT6037</td>
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<tr>
<td>Streptomyces griseus ISP5236</td>
</tr>
<tr>
<td>Streptomyces ipomoeae MAFF4023</td>
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<tr>
<td>Streptomyces plicatus ATCC 25483</td>
</tr>
<tr>
<td>Streptomyces prasinopilosus ISP3098</td>
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<tr>
<td>Streptomyces scabies MAFF4018</td>
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<tr>
<td>Streptomyces sp. S15</td>
</tr>
<tr>
<td>Streptomyces sp. S84</td>
</tr>
<tr>
<td>Streptomyces sp. S100</td>
</tr>
<tr>
<td>Streptomyces sp. S159</td>
</tr>
<tr>
<td>Bacillus circulans WL-12</td>
</tr>
<tr>
<td>Serratia marcescens 2170</td>
</tr>
<tr>
<td>Serratia marcescens QMB1466</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection; MAFF, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, Japan. † Isolated from soil by K. Miyashita.
Chemical Co. by the methods described by Yamada & Imoto (1981) and Jeuniaux (1966), respectively. Chitin EX (powdered prawn shell chitin) was purchased from Funakoshi Chemical Co. Regenerated chitin was prepared from chitosan 8B (80% deacetylated chitin) purchased from Funakoshi Chemical Co. as described by Molano et al. (1977). Soluble chitin was obtained from Yaizu Suisan Chemical Co. The degree of deacetylation and approximate molecular mass of the soluble chitin were 38.8% and 20–30 kDa, respectively. Allosamidin was purified from *Streptomyces* sp. 1713 by extraction with methanol and then column chromatography with active carbon, Dowex-50 and SP-Sephadex C-25 by a method described previously (Sakuda et al., 1986).

Results

Production of *S. griseus* chitinase C in *E. coli*, and its purification

The *chiC* gene encoding chitinase C of *S. griseus* HUT6037 was originally cloned in *E. coli* JM109 as the recombinant plasmid pGC01 carrying a 1.7 kb insert in pUC119 (Ohno et al., 1996). Since no chitinase production was observed in *E. coli* cells carrying this plasmid, the regions upstream of the deduced promoter sequence and downstream of the probable terminator sequence of the *chiC* gene were removed from the insert of pGC01, and the resulting fragment was used as the insert in plasmid pGC01A. *E. coli* cells carrying pGC01A produced chitinase C, but the amount produced was still not sufficient to carry out biochemical and structural studies (data not shown). Therefore, to achieve higher expression, the coding region of the *chiC* gene was amplified by PCR and ligated to *NdeI*- and *BamHI*-cut pET12a, yielding the expression plasmid pGC02. *E. coli* BL21(DE3) cells carrying pGC02 produced much higher levels of chitinase C, which was accumulated in the periplasmic space.

Chitinase C was extracted from the periplasmic space of BL21(DE3) cells and precipitated with ammonium

![Fig. 1. (a) Purification of chitinase C by hydroxylapatite column chromatography. Crude chitinase C preparation was dissolved in a small volume of 20 mM phosphate buffer (pH 6.0) and applied onto a hydroxylapatite column previously equilibrated with the same buffer. Proteins were eluted with the same buffer and elution was monitored by absorbance at 280 nm. The peak fractions indicated by the bold bar containing purified chitinase C were collected. (b) SDS-PAGE analysis of purified chitinase C. Lanes: 1, proteins (200 µg) in the culture supernatant of *S. griseus* HUT6037; 2, periplasmic proteins (50 µg) prepared from *E. coli* BL21(DE3) carrying pGC02; 3, purified chitinase C (20 µg). Proteins were stained with Coomassie brilliant blue R-250.](image)

**Table 2.** Specific activities of chitinase C (U mg⁻¹) on various chitinous substrates and comparison with those of other bacterial chitinases

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>S. griseus</em> ChiC</th>
<th><em>Bacillus circulans</em> ChiA1</th>
<th><em>Bacillus circulans</em> ChiC1</th>
<th><em>Serratia marcescens</em> ChiA</th>
<th><em>Serratia marcescens</em> ChiB</th>
<th><em>Serratia marcescens</em> ChiC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal chitin</td>
<td>2450</td>
<td>4.19</td>
<td>3.19</td>
<td>4.06</td>
<td>4.35</td>
<td>3.89</td>
</tr>
<tr>
<td>Glycol chitin</td>
<td>255.02</td>
<td>0.76</td>
<td>20.90</td>
<td>25.40</td>
<td>1.46</td>
<td>2.40</td>
</tr>
<tr>
<td>Regenerated chitin</td>
<td>1.84</td>
<td>1.44</td>
<td>0.38</td>
<td>0.62</td>
<td>0.48</td>
<td>0.40</td>
</tr>
<tr>
<td>Squid chitin</td>
<td>0.19</td>
<td>3.26</td>
<td>0.54</td>
<td>0.90</td>
<td>0.12</td>
<td>0.45</td>
</tr>
<tr>
<td>Chitin EX</td>
<td>0.16</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Chitosan 7B</td>
<td>9.73</td>
<td>5.93</td>
<td>18.90</td>
<td>3.14</td>
<td>3.95</td>
<td>2.94</td>
</tr>
<tr>
<td>Chitosan 8B</td>
<td>8.06</td>
<td>4.47</td>
<td>16.80</td>
<td>1.78</td>
<td>2.90</td>
<td>2.27</td>
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<tr>
<td>Chitosan 9B</td>
<td>0.68</td>
<td>0.41</td>
<td>2.11</td>
<td>0.02</td>
<td>0.82</td>
<td>0.67</td>
</tr>
<tr>
<td>Chitosan 10B</td>
<td>0.20</td>
<td>0.02</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Enzymic properties and antifungal activity of chitinase C

The enzyme was stable at up to 55°C during a 15 min incubation at pH 6.0. Approximately 10 mg purified chitinase C was obtained from a 1 litre culture of *E. coli* BL21(DE3) carrying pGC02.

The hydrolysing activity of chitinase C against various chitinolytic bacteria was determined using colloidal chitin as the assay substrate. Chitinase C maintained almost the same level of activity from pH 4 to 8.5; it showed practically no activity at pH values below 2.5 or over 10.5. When measured at pH 6.0, maximum activity was observed at around 55°C. The enzyme was stable at up to 55°C during a 15 min incubation at pH 6.0.

The hydrolysing activity of chitinase C against various chitinolytic bacteria was studied and compared with those of family 18 chitinases. As shown in Table 2, chitinase C of *S. griseus* HUT6037 exhibited the highest activity against glycol chitin among the tested chitinolytic bacteria. It hydrolysed all other substrates much less efficiently: hydrolysing activity against colloidal chitin, for example, was one-tenth of that activity against glycol chitin. However, when compared with other bacterial chitinases family 18, the specific hydrolysing activity of chitinase C was remarkably high against both soluble and insoluble chitin, including colloidal chitin, glycol chitin and powdered chitin (chitin EX).

Plant chitinases are thought to act as a part of the defence mechanism against plant-pathogenic fungi, and antifungal activity of plant chitinases from various sources, especially family 19 chitinases, has been demonstrated by many researchers (Broekaert *et al.*, 1988; Leah *et al.*, 1991; Schlumbaum *et al.*, 1986; Roberts & Selitrennikoff, 1988; Ishi *et al.*, 1993). On the other hand, bacterial chitinases are thought to be involved mainly in digestion of chitin for nutritional purposes, and are reported not to be effective with respect to antifungal activity (Roberts & Selitrennikoff, 1988). However, the bacterial chitinases tested to date for antifungal activity have all belonged to family 18. On the other hand, the plant chitinases having high antifungal activity belong to family 19. Therefore, we were very interested in whether bacterial family 19 chitinases have antifungal activity. The antifungal activity of chitinase C was tested as the inhibitory effect on the hyphal extension of *T. reesei* colonies formed on PDA plates. As shown in Fig. 2, 20 µg chitinase C clearly inhibited hyphal extension of *T. reesei*. The effect of allosamidin on the hydrolysing activity of chitinase C toward colloidal chitin was examined and compared with the effect on bacterial family 18 chitinases. No inhibition was observed even in the presence of 50 µg allosamidin ml⁻¹ in a reaction mixture which contained 50 µg chitinase C ml⁻¹ and 0.13% (w/v) colloidal chitin. On the other hand, the activities of the bacterial chitinases belonging to family 18 were severely inhibited by the presence of allosamidin. For example, the IC₅₀ of chitinase A1 from *B. circulans* WL-12 under similar reaction conditions was 0.27 µg ml⁻¹. Therefore, it was concluded that chitinase C is insensitive to allosamidin. The results are consistent with previously reported observation that yam chitinase, a plant family 19 chitinase, was not inhibited by allosamidin (Koga *et al.*, 1987).
Detection of allosamidin-resistant chitinases in the culture supernatants of various Streptomyces species

S. griseus chitinase C produced in E. coli was not inhibited by allosamidin, whereas the bacterial family 18 chitinases were severely inhibited. This meant that family 19 chitinases similar to chitinase C could be detected specifically as chitinases which were active in the presence of allosamidin. Therefore, in order to study whether family 19 chitinase is unique to S. griseus HUT6037 or common to Streptomyces species, we first attempted to detect allosamidin-insensitive chitinases in the culture supernatants of various Streptomyces species.

Streptomyces species were grown for 72 h in the presence of 0.2% (w/v) colloidal chitin and proteins in the culture supernatants were collected and subjected to SDS-PAGE analysis. After renaturation of proteins in the polyacrylamide gel, chitinase activity was detected by zymogram analysis in the presence and absence of allosamidin. The presence of allosamidin during the course of zymogram analysis was expected to reduce the intensity of activity bands or erase the activity bands of family 18 chitinases on the agar replica of the SDS-PAGE gel. As shown in Fig. 3(a), in the absence of allosamidin, several chitinase bands with a variety of sizes were detected in the culture supernatants of all Streptomyces species. On the other hand, as shown in Fig. 3(b), larger chitinase bands disappeared or were weakened drastically in the presence of allosamidin, and only one or two chitinase band(s) with sizes similar to that of chitinase C of S. griseus remained unaffected. The chitinase band in lane 1 of Fig. 3(b) corresponds to chitinase C1 (27 kDa), a proteolytic derivative of chitinase C (32 kDa) lacking its N-terminal chitin-binding domain. These results strongly suggest that all Streptomyces species tested in this study produce family 19 chitinases with sizes similar to that of S. griseus chitinase C in addition to family 18 chitinases.

Amplification of a portion of genes encoding family 19 chitinases

The sizes of the chitinases insensitive to allosamidin detected in the culture supernatants were very similar to that of chitinase C. To see whether Streptomyces species have genes similar to the chiA gene encoding chitinase
Family 19 chitinases of *Streptomyces*

Fig. 5. Partial amino acid sequences of family 19 chitinases of various *Streptomyces* species deduced from the nucleotide sequences of PCR-amplified DNA fragments. Amino acids identical to those of the corresponding sequences of *S. griseus* chitinase C are represented by dots. 1, Corresponding region of *S. griseus* HUT6037 chitinase C; 2, amplified fragment 1 from *S. coelicolor*; 3, fragment 1 from *S. lividans* 66; 4, fragment 2 from *S. lividans* 66; 5, fragment 3 from *S. lividans* 66; 6, fragment 1 from *S. coelescens*; 7, fragment 2 from *S. coelescens*; 8, fragment 1 from *S. ipomoeae*; 9, fragment 1 from *S. prasinopilus*; 10, fragment 2 from *S. prasinopilus*; 11, *Streptomyces* sp. S15; 12, *Streptomyces* sp. S84; 13, *Streptomyces* sp. S100; 14, *Streptomyces* sp. S159.

Fig. 6. Comparison of the partial sequences of *Streptomyces* family 19 chitinases with plant chitinases. Sequence alignment was done using the CLUSTAL X program. GenBank accession numbers of plant chitinases used in this analysis: X15494, potato class I; X16938, tobacco class I; X56063, rice class I; X51427, petunia class II; M62904, barley class II; L25826, beet class IV; X61488, rape class IV; M84164, maize class IV.

C, Southern hybridization experiments using a part of the *chiC* gene as a probe were carried out. Chromosomal DNA was extracted from various *Streptomyces* species listed in Table 1 and digested with *Sal* I and *Pst* I. The probe used in the experiments contained the region corresponding to the entire catalytic domain of chitinase C plus the adjacent 200 bp region downstream from the termination codon. As shown in Fig. 4, strong signals were observed with all *Streptomyces* species tested. The patterns of the detected signals were identical among *S. coelicolor* A3(2), *S. lividans*, *S. eurythermal*, *S. cyanocolor*, *S. scabies*, *S. plicatus*, *S. lividans* 66, *S.
coelestes, and between S. ipomoeae and S. flavovirens. Some of the patterns contained two signals and others only one when chromosomal DNAs were digested with Sall (left lanes of each set). These results strongly suggest that all Streptomyces species examined here possess family 19 chitinase gene(s) similar to chiC and many of them may have more than one such gene.

To confirm the above results and to obtain sequence information about the putative family 19 chitinase genes of these Streptomyces species, we attempted to amplify a region of these genes by PCR. To design PCR primers, the amino acid sequences of S. griseus chitinase C and plant class I, II and IV chitinases were compared, and the two most-conserved regions were deduced. Forward and reverse primers were designed corresponding to these amino acid regions, from Lys-134 to Ala-142 and from Ile-256 to Cys-262, respectively, of chitinase C. Chromosomal DNAs from Streptomyces species which gave different patterns of hybridization signals were used as templates for PCR. DNA fragments approximately 400 bp in length were amplified for all Streptomyces species used. These fragments were ligated with pT7Blue vector and their nucleotide sequences were determined. More than two fragments with some differences in the nucleotide sequences were obtained from some Streptomyces species, suggesting multiple genes for family 19 chitinase. Fig. 5 shows amino acid sequences deduced from the nucleotide sequences of the amplified DNA fragments. All of the sequences were very similar not only to each other but also to the corresponding region of chitinase C. The mean amino acid and nucleotide identities of the sequences, including the corresponding region of chitinase C, were 87.3% and 87.2%, respectively. Therefore, all Streptomyces species tested in this study possess genes (or at least a portion of the gene) for family 19 chitinases which are very similar to the gene for chitinase C of S. griseus HUT6037.

In order to compare family 19 chitinases of Streptomyces species with those of plants, the amino acid sequences deduced from the amplified fragments were aligned with the amino acid sequences of the corresponding regions of plant family 19 chitinases. As shown in Fig. 6, family 19 chitinases of Streptomyces appeared to have deletions at the two positions, when compared with class I chitinases. Surprisingly, the positions of the deletions observed in Streptomyces chitinases are exactly the same as those observed in class IV chitinases, although the second deletions in Streptomyces are a little shorter than those observed in class IV chitinases. Therefore, in terms of the presence and positions of deletions of amino acid segments, Streptomyces family 19 chitinases can be classified as class IV type.

**DISCUSSION**

Genes for family 19 chitinases very similar to chitinase C of S. griseus HUT6037 were detected in all Streptomyces species examined in the present study. Family 19 chitinases had only been reported from higher plants until chitinase C of S. griseus HUT6037 was discovered. Plant chitinases are divided into six classes based on their amino acid sequence features (Collinge et al., 1993). Among them, classes I, II and IV belong to family 19, whereas classes III and V belong to family 18. Classes I and IV have a cysteine-rich domain at their N-termini which is involved in chitin binding. Class II chitinases lack such a domain. The catalytic domains of class IV chitinases have deletions of several amino acids at two positions as compared with those of class I and II chitinases, and this is the criterion for distinguishing class IV from class I chitinases. In the present study, we demonstrated that Streptomyces species commonly possesses family 19 chitinases which are class IV type in the sense that they have deletions at the same positions as class IV chitinases do. More than two distinct fragments with different nucleotide sequences were amplified by PCR, indicating multiple genes for family 19 chitinase. Presence of multiple family 19 genes may be common in Streptomyces species. Indeed, we have shown the presence of two distinct genes for family 19 chitinases located within 600 kb of both ends of the S. coelicolor chromosome (Saito et al., 1999). The chiF gene encodes a family 19 chitinase with similar domain structure to S. griseus chitinase C, consisting of an N-terminal chitin-binding domain and a C-terminal catalytic domain. On the other hand, a family 19 chitinase encoded by the chiG gene is devoid of an N-terminal chitin-binding domain and thus consists of only a catalytic domain. A part of the gene obtained by PCR from the S. coelicolor chromosomal DNA in the present study corresponded to a part of the chiF gene.

To visualize the relationship among Streptomyces family 19 chitinases and plant family 19 chitinases in terms of amino acid sequence similarity, a phylogenetic tree was constructed (Fig. 7). To avoid the effects of deletions, the amino acid segments of family 19 chitinases corresponding to positions 53–102 as indicated in Fig. 6 were used to make the sequence alignment and construct the tree. Class I/II chitinases and Streptomyces chitinases each formed a discrete cluster, whereas class IV chitinases were rather dispersed throughout the tree. The divergency of Streptomyces chitinases was somewhat lower than that among class I/II chitinases and markedly lower than that among class IV chitinases. Two possible evolutionary relationships of Streptomyces family 19 chitinases with plant chitinases can be imagined. One is that a common ancestral chitinase was already present prior to divergence of plant and bacteria, and family 19 chitinases then evolved independently in plants and bacteria. In this case, class IV type is the original form of family 19 chitinases. This idea does not seem to be likely because amino acid sequence similarities among Streptomyces family 19 chitinases and certain plant chitinases are extremely high. For example, the amino acid identity between the catalytic domain of S. griseus HUT6037 chitinase C (213 amino acids) and that of maize chitinase is 44%. The other possibility, which is more likely to be the case, is that family 19 Streptomyces chitinases were
acquired from plants by horizontal gene transfer. *Streptomyces* species are important soil microorganisms, and higher plants and *Streptomyces* share a common habitat in many places. In addition, some strains of *Streptomyces* are plant pathogens; *S. scabies* and *S. ipomoeae* included in the present study are examples of such *Streptomyces*. Such a close relationship, including direct interactions between *Streptomyces* and higher plants, would increase the chances for horizontal gene transfer from plants to *Streptomyces*.

The high sequence similarity among *Streptomyces* family 19 chitinases and the limited distribution of family 19 chitinases in prokaryotic organisms appear to support the idea that the acquisition of family 19 chitinases by *Streptomyces* occurred relatively recently. Hamel *et al*. (1997) suggested that classes I and IV were derived from a common ancestral sequence that predates the divergence of dicots and monocots. It is not clear whether the gene transfer event occurred before or after divergence of class IV and class I/II chitinases. More detailed evolutionary analysis will be required to clarify this point.

As demonstrated in the present study, *S. griseus* chitinase C exhibited strong antifungal activity, whereas the other bacterial chitinases which belong to family 18 did not. Plant chitinases are thought to be a part of the defence mechanism against fungal pathogens, and the antifungal activity of plant family 19 chitinases (class I, II and IV) has been demonstrated by many researchers (Broekaert *et al*., 1988; Leah *et al*., 1991; Schlumbaum *et al*., 1986; Roberts & Selitrennikoff, 1988; Iseli *et al*., 1993). This fact led us to test the antifungal activity of *S. griseus* chitinase C. Chitinase C exhibited a remarkable ability to inhibit hyphal extension of *T. reesei*. These results imply that antifungal activity may be a general property of family 19 chitinases. Chitinase C has a chitin-binding domain at its N-terminus, but the amino acid sequence features of this domain show no relationship to those of chitin-binding domain of plant class I and IV chitinases. Iseli *et al*. (1993) reported that the chitin-binding domain (cysteine-rich domain) of tobacco class I chitinase was essential for chitin binding but not for antifungal activity. Deletion of the cysteine-rich domain reduced the antifungal activity, and therefore this domain was suggested to enhance the antifungal activity of this chitinase.

*B. circulans* chitinase A1, which has a chitin-binding domain similar to that of chitinase C, did not show any ability to inhibit hyphal extension. Therefore, the chitin-binding domain of chitinase C itself probably does not have antifungal activity, and the catalytic domain probably makes the major contribution to the observed antifungal activity. However, it will be very interesting to see whether the chitin-binding domain of chitinase C has the function of improving the antifungal activity of this enzyme, as demonstrated for the tobacco class I chitinase. Construction of an expression system for the catalytic domain, to test whether the loss of the chitin-binding domain of chitinase C reduces the antifungal activity, is under way. Such efforts may give us a tool for improving the antifungal activity of chitinases by adding or altering the chitin-binding domains of antifungal chitinases.

The general occurrence of family 19 chitinases in *Streptomyces* was demonstrated in this study. Our latest
results suggest the presence of family 19 chitinases in a few bacterial species in addition to Streptomyces (data not shown). This means that family 19 chitinases are relatively rare in prokaryotic organisms, but not restricted only to Streptomyces species. These chitinases are expected to have different properties in different prokaryotic organisms, including the strength and specificity of the antifungal activity. Therefore, prokaryotic organisms may be a good source of highly active chitinases and chitin-binding domains with respect to antifungal activity and efficiency of chitin degradation.

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