Induction and Repression of a *Streptomyces lividans* Chitinase Gene Promoter in Response to Various Carbon Sources

Kiyotaka Miyashita,† Takeshi Fujii, and Akihiro Saito

National Institute of Agro-Environmental Sciences, 3-1-1 Kan-nondai, Tsukuba 305-8604, Japan

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Induction and repression of a gene for chitinase (chiA) in *Streptomyces lividans* was investigated using a catechol 2,3-dioxygenase gene (*xylE*) as the reporter gene. Of various substrates examined, expression of the promoter (PchiA) was observed after a delay when colloidal chitin or small chitin-oligosaccharides were added to the medium. N-acetylglucosamine completely repressed the chiA promoter. The duration of the delay in expression of PchiA differed with the inducer used, with chitobiase inducing the activity most rapidly. The minimum concentration of chitobiase needed for induction was 1 μM. It appears, therefore, that an efficient inducer of the gene for chitinase in *S. lividans* is chitobiase.

Key words: *Streptomyces lividans*; chitinase gene; catabolite repression; chitobiase

*Streptomyces* spp. are major producers of chitinases in soil and they use chitin as their source of carbon. Synthesis of the chitinases of *Streptomyces* spp. is induced by chitin, and is repressed in the presence of readily usable carbon sources such as glucose. Genes for chitinase have been cloned from *Streptomyces plicatus,* *Streptomyces olivaceoviridis,* *Streptomyces lividans,* *Streptomyces coelicolor,* *Streptomyces thermoviolaceus,* and *Streptomyces griseus.* Chitinases of *Streptomyces* are multiple enzymes, and we reported the high multiplicity of chitinase genes in *Streptomyces coelicolor.* Chitinase synthesis of *Streptomyces* is induced by chitin, and repressed by readily usable carbon sources such as glucose. From the analysis of expression of chi63 and chi35 from *S. plicatus* and chiA and chiC from *S. lividans,* it has been shown that chitinase synthesis is regulated at the level of transcription. In the promoter regions of the chitinase genes of *Streptomyces,* a pair of direct repeat sequences is commonly found, and it has been indicated to facilitate both chitin induction and glucose repression. The presence of the same direct repeat sequence in the promoters of the chitinase genes from different species suggests that a common regulatory mechanism is involved in the expression of chitinase genes of *Streptomyces.*

While insoluble chitin is generally used to induce chitinase in *Streptomyces,* it is not known whether chitin itself or one of its hydrolysis products, i.e., soluble chitin oligomer or N-acetylglucosamine, acts as the inducer of the chitinase genes. In this study, the expression of a chitinase gene (chiA) promoter in *S. lividans* in response to various substrates was investigated.

Materials and Methods

*Bacterial strains, plasmids, and culture conditions.* The strains of *Streptomyces* and *Escherichia coli* used were *S. lividans* strain TK24 and *E. coli* JM109, respectively. *E. coli* JM109, harboring pUC18, pUC19, or a derivative of their plasmids, was grown on LB or SOC medium with ampicillin (100 μg/ml). *S. lividans* that harbored pEMX151 was grown in LB or inorganic-salts medium with thiostrepton (50 μg/ml; Sigma, St. Louis). A series of small chitin-oligosaccharides, chitobiase (di-N-acetyl-chitobiase) to chitohexaose (hexa-N-acetyl-chitohexaose), and chitosan dimer were purchased from Seikagaku Kogyo (Tokyo). Carbohydrate use was studied by the method of Shirling and Gottlieb.

*Recombinant DNA techniques.* Preparation of protoplasts, transformation, and selection of transformants of *Streptomyces* were done by the methods of Hopwood et al. All procedures involving recombinant DNA were performed as described by Sambrook et al.

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† To whom correspondence should be addressed. K. Miyashita, Tel: +81-298-38-8256; Fax: +81-298-38-8199; E-mail: kmiyas@nias.aflrc.go.jp

Abbreviations: chiA, a gene for chitinase A of *S. lividans*; xylE, a gene for catechol 2,3-dioxygenase of *Pseudomonas putida*; PchiA, a promoter of chiA; 4-MU, 4-methylumbelliferyl
from *S. lividans*, the *chiA* gene\(^5\) was used for the study of its promoter. A *PvuII* fragment of *chiA*, containing about 500-bp regions both upstream and downstream from the site of initiation of transcription, was cloned into pUC18. The fragment, recovered by digestion with *HindIII* and *BamHI*, was ligated into the *HindIII* and *BamHI* sites of pXE4 upstream of *xyle*.\(^1\) The resulting plasmid, designated pEMX151, was introduced into *S. lividans* TK24 by transformation. The process of construction is illustrated in Fig. 1.

Assays of *Xyle* and chitinase activities. *S. lividans* TK24 harboring pEMX151 was grown in LB medium with thiostrepton (50 \(\mu\)g/ml) for 40 hours at 30°C. Cells were harvested by centrifugation, washed with inorganic-salts medium, and resuspended in inorganic-salts medium with various carbon sources and thiostrepton. Cells were harvested from 1-ml portions of the culture at various times, disrupted by sonication, and *xyle* gene products were measured by the method of Sala-Trepat et al.\(^8\) *Xyle* activity was calculated as the rate of change in optical density at 375 nm per minute per milligram of protein and converted to milliunits per milligram.\(^10\)

Chitinase activity in culture filtrates was measured as described previously,\(^6\) with the fluorogenic substrate 4-methylumbelliferyl N,N’-diacetyl chitobiose as the substrate. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmole of 4-MU from the substrate in one minute at 37°C.

### Results and Discussion

**Induction of *chiA* promoter in response to various substrates**

Among the three genes for chitinases cloned from *S. lividans*, the *chiA*\(^5\) gene was used for study of its promoter. Our previous study showed that chitinase A was one of the major chitinases produced by the bacterium. The expression of *chiA* was induced by chitin and repressed by glucose, and the expression of *chiA* was directed from its promoter.\(^5\) A site of initiation of transcription was found approximately 51 bp upstream from the GTG initiation codon. A pair of 12-bp direct repeat sequences similar to those of a chitinase gene of *Streptomyces plicatus*,\(^11\) was found to overlap the binding site of RNA polymerase in the *chiA* promoter. The importance of the direct repeat sequences in the regulated expression of *chiA* was indicated.\(^5\)

pEMX151 was constructed as shown in Fig. 1, and introduced into *S. lividans* by transformation. In this plasmid, the promoterless *xyle* cassette lies 0.5 kb downstream of the GTG start codon of *chiA*, and catechol 2,3-dioxygenase (*Xyle*) activity reflects *chiA* expression. Since pXE4 is a low copy plasmid vector,\(^17\) the effect of copy number of *chiA* can be considered to be minimal.

Induction of the *chiA* promoter by colloidal chitin was investigated. Xyle activity in cell lysates was observed 4 hours after addition of colloidal chitin (0.25%, w/v; Fig. 2). The activity increased thereafter, and high-level expression of *Xyle* was observed after 10 hours. No chitinase activity in the culture filtrate, however, could be detected even 10 hours after addition of colloidal chitin, and more than one day was required before chitinase activity was detectable in the culture supernatant (data not
Expression of chIA Promoter in S. lividans

shown). This discrepancy was considered to be due to the chitin-binding properties of the enzymes.\(^5\)\(^5\)

Since chitinase produced early in growth was bound firmly to insoluble chitin, chitinase activity could not be detected in the culture supernatant. It was after digestion of colloidal chitin or release of the catalytic domain from the chitin-binding domain that the chitinase activity became detectable.

N-acetylglucosamine, small chitin-oligosaccharides, glucosamine, and various disaccharides including chitosan, chitobiose, and maltose, were assayed for their ability to induce transcription from the chIA promoter at concentrations of 1 mM. Of these compounds, chitobiose (di-N-acetylchitobiose), chitotriose (tri-N-acetylchitotriose), and chitotetraose (tetra-N-acetylchitotetraose) induced the chIA promoter while the monosaccharides and the other disaccharides did not (data not shown).

The effects of the concentration of chitobiose on the expression of chIA promoter were examined. Chitobiose of various concentrations was added to the medium, and XyIE activity was measured after 4 hours. As little as 1 μM chitobiose (0.00004%, w/v) induced the chIA promoter, and maximum activity was observed with 1 mM chitobiose (Fig. 3). Since the chitin-oligosaccharides induced the chIA promoter both in a shorter time and at a lower concentration than colloidal chitin did, it is suggested that the real inducer of chIA promoter is not insoluble chitin itself but soluble chitin-oligosaccharides.

Repression of the chIA promoter by various carbon sources

It is generally considered that several carbon sources other than glucose could cause catabolite repression in Streptomyces. We observed that glycerol completely suppressed chitinase synthesis in S. lividans.\(^6\) However, the repression of the chitinase gene in response to various carbon sources has been little studied. To examine the suppressive effects of carbon sources on chitinase-dependent induction of chIA promoter, S. lividans TK24 harboring pEMX151 cells were exposed to various carbon sources at a concentration of 50 mM as a supplement to 0.25% colloidal chitin, and XyIE activity was assayed after 10 hours. The carbon use test showed that S. lividans could use all these compounds as sole carbon sources. Of the substrates examined, glucose, N-acetylglucosamine, glycerol, arabinose, chitobiose, and fructose completely repressed the chIA promoter (Table 1). Other sugars including mannitol, glucosamine, and mannose decreased the repression of chIA promoter to less than 30% of the control, and galactose, maltose, xylose, and inositol had little influence on the expression. Between two disaccharides consisting of glucose, chitobiose, 4-(β-d-glucoside)-d-glucose, repressed the chIA promoter, while maltose, 4-(α-d-glucoside)-d-glucose did not. N-acetylglucosamine, an end product of chitin biodegradation and an inducer of chitinase production in several bacteria,\(^16\) repressed induction of the chIA promoter by colloidal chitin.

Comparison of the induction by various chitin oligosaccharides

Induction of chIA promoter by small chitin oligosaccharides (chitobiose, chitotriose, and chitotetraose) was compared. S. lividans harboring pEMX151 was grown in LB and the cells were transferred to inorganic-salts medium with 10 μM of one of the chitin-oligosaccharides to be tested. The cells were incubated at 30°C, and XyIE activity was measured every 30 minutes as described above.

Table 1. Carbon Catabolite Repression of PchIA in S. lividans

<table>
<thead>
<tr>
<th>Compound</th>
<th>Repression</th>
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<tbody>
<tr>
<td>None (control)</td>
<td>–</td>
</tr>
<tr>
<td>Glucose</td>
<td>+ +</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>+ +</td>
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<tr>
<td>Glycerol</td>
<td>+ +</td>
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<tr>
<td>Arabinose</td>
<td>+ +</td>
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<tr>
<td>Chitobiose</td>
<td>+ +</td>
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<tr>
<td>Fructose</td>
<td>+ +</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
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<tr>
<td>Glucosamine</td>
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<tr>
<td>Mannose</td>
<td>+</td>
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<tr>
<td>Galactose</td>
<td>–</td>
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<td>Maltose</td>
<td>–</td>
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<tr>
<td>Xylose</td>
<td>–</td>
</tr>
<tr>
<td>Inositol</td>
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</table>

S. lividans TK24 harboring pEMX151 was grown in 0.25% colloidal chitin medium with or without additional carbon sources, and XyIE activity was measured. Activity in media with additional carbon sources is given as a percentage of the control (colloidal chitin medium only).

+ +, completely repressed (XyIE activity was not detected); +, repressed (XyIE activity was less than 30% of fully induced level); –, not repressed (XyIE activity was more than 50% of fully induced level).

Fig. 3. Effects of Concentration of Chitobiose on the Induction of Catechol 2,3-dioxygenase Synthesis. S. lividans with pEMX151 grown in LB were transferred to inorganic-salts medium with various concentration of chitobiose, and XyIE activity was measured after 4 hours. The figures represent the averages and standard deviations of three independent measurements.
While induction of the chiA promoter by chitobiose was detected after 2 hours, induction by either chitotriose or chitotetraose was apparent only after 3 hours (Fig. 4).

chiA is not the sole chitinase gene in S. lividans, and chiA, chiB, and chiC encode different chitinase activities. When soluble chitin oligosaccharides were used as inducers, it was possible to measure the chitinase activity of the culture supernatant even early in growth. To see if the expression of the chiA promoter reflects total chitinase activity of the bacterium, the chitinase activity of the culture supernatant was measured. Since S. lividans grew faster in the absence of thiostrepton in the medium, the length of the first culture was shortened to 20 hours. The S. lividans TK24 cells were then exposed to various chitin oligosaccharides (100 μM), and chitinase activity in the culture filtrate was measured every 20 min. Chitinase activity caused by chitobiose increased more rapidly than that by either chitotriose or chitotetraose (Fig. 5). Similar results were obtained when the concentration of chitin oligosaccharide was decreased to 10 μM. Induction by chitopentaose or chitohexaose also occurred after a longer delay than that by chitobiose (data not shown). It was thus indicated that the expression of chiA reflects the induction of total chitinase enzymatic activity in S. lividans. In spite of the diversity of the predicted amino acid sequences among these genes in S. lividans, a similar direct repeat sequence exists in the promoter of all the chitinase genes analyzed. It is, therefore, suggested that these genes are regulated coordinately. Concurrent expression of the multiple chitinase genes was observed in S. coelicolor (Saito et al., unpublished data).

These results showed that chitobiose was the most effective inducer among the small chitin oligosaccharides tested with regard to duration of the delay in the expression of activity. The prolonged delay with the chitin oligosaccharides other than chitobiose might reflect the time needed to generate chitobiose by the action of chitinases, which is produced constitutively at a low level. The possibility that chitin oligosaccharides other than chitobiose are also inducers of the chitinase gene cannot be excluded, even if they are less competent than chitobiose. However, it seems reasonable that chitobiose is a real inducer of chitinase genes in S. lividans, since chitobiose is a major product of the hydrolysis of chitin by the extra-cellular chitinases of Streptomyces. It is surprising that as low as 1 μM (0.00004%) chitobiose functioned as an inducer of the chitinase gene. Hence it is not likely that the metabolism of chitobiose is involved in the induction of the gene.

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References


