Transcriptional co-regulation of five chitinase genes scattered on the *Streptomyces coelicolor* A3(2) chromosome

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***Streptomyces coelicolor* A3(2) strain M145 has eight chitinase genes scattered on the chromosome: six genes for family 18 (*chiA*, *B*, *C*, *D*, *E* and *H*) and two for family 19 (*chiF* and *G*). In this study, the expression and regulation of these genes were investigated. The transcription of five of the genes (*chiA*, *B*, *C*, *D* and *F*) was induced in the presence of colloidal chitin while that of the other three genes (*chiE*, *G* and *H*) was not. The transcripts of the five induced *chi* genes increased and reached their maximum at 4 h after the addition of colloidal chitin, all showing the same temporal patterns. The induced levels of the transcripts of *chiB* were significantly lower than those of the other four genes. Dynamic analysis of the transcripts of the *chi* genes indicated that *chiA* and *chiC* were induced more strongly than *chiD* and *chiF*. Addition of chitobiose also induced transcription of the *chi* genes, but significantly earlier than did colloidal chitin. When cells were cultured in the presence of colloidal chitin, an exponential increase of chitobiose concentration in the culture supernatant was observed prior to the induced transcription of the *chi* genes. This result, together with the immediate effect of chitobiose on the induction, suggests that chitobiose produced from colloidal chitin is involved in the induction of transcription of the *chi* genes. The transcription of the five *chi* genes was repressed by glucose. This repression was apparently mediated by the glucose kinase gene *glkA*.**

Keywords: chitin, streptomycetes, transcriptional regulation, glucose repression, chitobiose

INTRODUCTION

Chitin is the second most abundant polysaccharide after cellulose in nature. The decomposition of chitin, therefore, is important in terms of the carbon and nitrogen cycles in the biosphere. *Streptomyces* are saprophytic soil bacteria known as major decomposers of chitin in soil. The genes encoding chitinases (EC 3.2.1.14) – enzymes that hydrolyse chitin – have been cloned from them (Robbins *et al.*, 1988; Miyashita *et al.*, 1991, 1997; Blaak *et al.*, 1993; Fujii & Miyashita 1993; Miyashita & Fujii, 1993; Tsujibo *et al.*, 1993, 2000; Ohno *et al.*, 1996; Saito *et al.*, 1999). The products of these *Streptomyces* chitinase genes are classified, based on amino acid sequences analysis, into two distinct families of glycosyl hydrolases, families 18 and 19 (Henrissat, 1991; Henrissat & Bairoch, 1993). Family 19 chitinases also differ from family 18 chitinases in their

catalytic mechanisms (Armand *et al.*, 1994; Iseli *et al.*, 1996). *Streptomyces* are unique micro-organisms in possessing family 19 chitinase genes, which are usually found only in higher plants (Ohno *et al.*, 1996; Saito *et al.*, 1999; Watanabe *et al.*, 1999).

We previously cloned seven chitinase genes from the ordered cosmid library representing the entire chromosome of *S. coelicolor* A3(2) (Redenbach *et al.*, 1996) and analysed their sequences (Saito *et al.*, 1999). The genes do not form clusters but are scattered on the chromosome. Recently, the *S. coelicolor* A3(2) Genome Sequencing Project revealed the presence of another putative chitinase gene on cosmid 7B7. The accession and gene numbers are AL009199 and 7B7.09c, respectively. We named this gene *chiH*. Analysis of the deduced amino acid sequence of the gene product showed that ChiH belongs to subfamily C of bacterial family 18

Fig. 1. *S. coelicolor* A3(2) chitinase genes and the multiple domain structures of their deduced products. Arrows with open heads indicate the regions used for the preparations of labelled anti-sense RNAs. Similarity levels of DNA sequences between regions encoding the same domains are shown. Open boxes, checked boxes, boxes with arrows and striped boxes indicate regions encoding signal sequences, substrate-binding domains, fibronectin type III-like domains and catalytic domains, respectively. Each domain was predicted from its amino acid sequence.

chitinases (Suzuki *et al.*, 1999) and is presumed to consist of three domains: signal domain, catalytic domain and chitin-binding domain (Fig. 1). With the inclusion of *chiH*, *S. coelicolor* A3(2) evidently has genes encoding chitinases belonging to each subfamily of bacterial family 18 chitinases (Suzuki *et al.*, 1999), i.e. subfamily A (*chiC*, *chiD* and *chiE*), B (*chiA* and *chiB*) and C (*chiH*), in addition to the family 19 chitinases (*chiF* and *chiG*). The multiplicity of chitinase genes in *S. coelicolor* A3(2) is quite high as compared with other chitin-degrading bacteria, such as *Bacillus circulans* (Watanabe *et al.*, 1990, 1992) and *Serratia marcescens* (Watanabe *et al.*, 1997; Suzuki *et al.*, 1999).

Previous studies have shown that the transcription of the family 18 chitinase genes from *Streptomyces* is induced in the presence of chitin and repressed in the presence of glucose plus chitin (Delic *et al.*, 1992; Fujii & Miyashita, 1993; Miyashita & Fujii, 1993). We recently demonstrated that the transcription of *chiA*, a family 18 chitinase gene of *Streptomyces lividans*, was induced not only by colloidal chitin but also by chitin oligosaccharides, among which *N,N'*-diacetylchitobioside (chitobiose) was the most efficient inducer (Miyashita *et al.*, 2000).

Although the molecular mechanisms governing the regulation of expression of chitinase genes in *Streptomyces* are still unclear, a pair of direct repeat sequences present in the promoter region of *chi63*, a family 18 chitinase gene of *Streptomyces plicatus*, was shown to be involved in the induction by chitin and the repression by glucose of the gene expression (Delic *et al.*, 1992; Ni

& Westpheling, 1997). On the other hand, the *glkA* gene encoding a glucose kinase (Angell *et al.*, 1992) was shown to be involved in the glucose repression of chitinase production in *S. lividans* (Saito *et al.*, 1998). It was also demonstrated that the *glkA* gene is involved in the glucose repression of several catabolic enzymes, including agarase (Angell *et al.*, 1992), glycerol kinase (Seno & Chater, 1983) and α -amylase (Virolle & Bibb, 1988). It was thus inferred that the *glkA* gene plays a central role in the glucose repression of gene expression in *Streptomyces*. However, Ingram & Westpheling (1995) reported that *glkA* is not required for the glucose repression of the *chi63* promoter in an *S. coelicolor* A3(2) *ccrA1* genetic background.

The current study was done to elucidate the regulation of the expression of the chitinase system in *S. coelicolor* A3(2) M145, by investigating the induction and repression of the transcription of each chitinase gene in this strain. The promoter regions of the genes were deduced after the determination of their transcriptional initiation sites. The expression level of each chitinase gene is discussed in relation to the structure of its promoter region.

METHODS

Bacterial strains and media. The wild strain *Streptomyces coelicolor* A3(2) M145 and its *glkA* mutant M480 were used (Hopwood *et al.*, 1985; Angell *et al.*, 1994). *Escherichia coli* XL-1 Blue (Sambrook *et al.*, 1989) was used as the host for gene manipulations. LB medium (Sambrook *et al.*, 1989) and LB supplemented with 50 μ g ampicillin ml^{-1} were used for the

culture of *S. coelicolor* A3(2) and *E. coli* transformants, respectively. The expression of chitinase genes in *S. coelicolor* A3(2) was induced by growing the cells in YE (Saito *et al.*, 1998) or an inorganic salts medium (Miyashita *et al.*, 1991) with or without 0.05% (w/v) colloidal chitin (Lingappa & Lockwood, 1962) or chitobiose (1 mM or 250 µM) (Seikagaku Kogyo).

Culture conditions. Spores (approx. 6×10^7) of *S. coelicolor* A3(2) strains formed on SFM agar medium (van Wezel *et al.*, 1997) were inoculated into 30 ml LB medium in a 100 ml flask with a spring (Hopwood *et al.*, 1985) and grown for 20 h at 30 °C on a rotary shaker at 150 r.p.m. Mycelia were harvested by centrifugation, washed with either YE or inorganic salts medium, resuspended, and divided into several aliquots. After centrifuging the aliquots, the mycelia were resuspended in the corresponding medium supplemented with various carbon sources, i.e. 0.05% (w/v) colloidal chitin, 1 mM or 250 µM chitobiose, or 0.05% (w/v) colloidal chitin with 1.0% (w/v) glucose. The culture was further grown at 30 °C on a rotary shaker at 150 r.p.m. Samples of 5 ml, taken periodically, were centrifuged to separate the supernatants and the mycelia and stored at -80 °C.

Recombinant DNA techniques. Plasmid preparation and restriction enzyme digestion were done as described by Sambrook *et al.* (1989). Dephosphorylation, blunting and ligation of DNA fragments were done using bacterial alkaline phosphatase (Toyobo), a DNA blunting kit (Takara) and a DNA ligation kit (Takara), respectively, according to the manufacturers' instructions.

Northern-blot hybridization. The mycelia of *S. coelicolor* A3(2) harvested from a 5 ml culture sample by centrifugation were disrupted by using alumina type A-5 (Sigma) and a pellet mixer (Treff Lab), then total RNAs were prepared using an RNeasy mini preparation kit (QIAGEN) according to the manufacturer's instructions. Two or three micrograms of total RNA were electrophoresed on agarose gels containing 0.22 M formaldehyde and blotted to a Hybond-N+ membrane (Amersham Pharmacia Biotech) following standard procedure (Sambrook *et al.*, 1989). The probes were prepared as follows. For *chiA*, *chiB*, *chiC*, *chiE*, *chiF* and *chiG*, the regions shown in Fig. 1 were obtained by digesting pCA01, pCB02, pCC01, pCE01, pCF101 and pCG101 (Saito *et al.*, 1999), respectively, with appropriate restriction endonucleases. For *chiD*, the downstream region of the gene was deleted from the inserted fragment of pCD201 (Saito *et al.*, 1999) by using a DNA deletion kit (Takara). The resulting plasmid was digested by restriction endonucleases to obtain the region shown in Fig. 1. For *chiH*, the region indicated in Fig. 1 was obtained by PCR with the total DNA of *S. coelicolor* A3(2) M145 as template, and the oligonucleotides 5'-ACCCCGCTGCCGGACCGGTCTTCG-3' and 5'-GCCCTGGGTCGCGGTTTGCGTGTCC-3' as primers, and the KOD Dash DNA polymerase (Toyobo).

The DNA fragments generated above were inserted into pSPT18 and used for the labelling of the anti-sense RNAs with digoxigenin using the *in vitro* DIG RNA labelling kit-SP6/T7 (Roche Diagnostics). Hybridization was done at 68 °C in an EasyHyb buffer (Roche Diagnostics) and the hybridized probes were detected using a DIG detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Calculation of doubling time of mRNA accumulation. The mRNA of the chitinase gene was detected by Northern-blot analysis as described above and the intensity of the detected signals was measured using the Diversity database (Protein Databases Inc.). The logarithms of the obtained values indicating the intensity were then plotted. When the por-

portion between the values and time was observed from $[a]$ to $[a+b]$ hours after exposure to inducers, the mean increasing ratio of the mRNA per hour $[R]$ can be given with the intensity of signals $[X_n]$ at time $[n]$ by the following formula:

$$R = 1/b \sum X_{n+1}/X_n (a \leq n \leq a+b)$$

The doubling time of the mRNA amount (T) can then be estimated as T (min) = 120/ R .

Primer extension. The transcription initiation sites of *chiB*, *chiD* and *chiF* were determined by primer extension analysis. The primers 5'-GGTCACGGTCGTGCCCCGCGGTACAG-3' (primer B), 5'-GTGAAGTAGCCGTGACCTTGGACC-3' (primer D) and 5'-GCCGACAGGCGGTGGTAGCTCGAAC-3' (primer F), whose 5'-ends were labelled with the fluorescent dye FITC, were used for the primer extension reactions of the *chiB*, *chiD* and *chiF* genes, respectively. Total RNA was prepared from mycelia grown for 2 h in the presence of 250 µM chitobiose as described above. Eleven micrograms of the total RNA and 1 pmol of each primer were denatured at 90 °C for 1 min and annealed at 60 °C for 2 min in 13 µl buffer (50 mM Tris/HCl pH 8.0, 100 mM KCl). Reverse transcription was done at 42 °C for 1 h with Super Script II RNaseH⁻ Reverse Transcriptase (Life Technologies) in the presence of dATP, dCTP, dTTP and 7-deaza-dGTP (Roche Diagnostics). Size ladders were produced by a dideoxy sequencing reaction of the plasmids pCB01, pCD101 and pCF101 (Saito *et al.*, 1999) with the primers B, D and F, respectively. Sequencing reactions were performed using a thermo-sequenase fluorescent-labelled primer cycling kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) following the manufacturer's instructions. The primer extension products and the size ladders were separated on a 4.0% (w/v) polyacrylamide gel containing 10% (v/v) formamide at 40 °C by an automated DSQ 2000L Laser Fluorescent Sequencer (Shimadzu).

Chitinase assay. Chitinase activity in the culture supernatant was measured as described previously (Miyashita *et al.*, 1991) using the fluorescent substrate 4-methylumbelliferyl-N,N'-diacetylchitobioside or 4-methylumbelliferyl-N,N',N'-tri-acetylchitotrioside (Sigma).

Determination of chitobiose concentration. Culture supernatant obtained by centrifugation was boiled for 10 min, cooled on ice, and filtered through a cellulose acetate membrane with pore size of 0.20 µm (Millipore). A 0.25 ml aliquot of the filtrate was mixed with 0.75 ml acetonitrile containing 100 ng ml⁻¹ of *p*-nitrophenyl-N,N',N'-tri-acetylchitotrioside [PNP-(GlcNAc)₃] as the internal standard and centrifuged at 17000 *g* at 4 °C for 10 min. The supernatant was injected into an HPLC-tandem mass spectrometer (MS/MS) for quantifying chitobiose concentration. The conditions for the separation of chitobiose were as follows: HPLC system, 1090 Series II (Hewlett Packard); column, Capcell Pac NH2 UG80A 5 mm, 1.5 × 150 mm (Shiseido); guard column, Opti-Guard C₁₈, 1.0 × 15 mm (Optimize Technologies); oven temperature, 40 °C; eluent, 75% (v/v) acetonitrile; and flow rate, 0.1 ml min⁻¹. The eluent passing the column was mixed with 75% (v/v) acetonitrile containing 200 mM acetic acid at the flow rate of 0.01 ml min⁻¹ and then led into an API300 triple-quadrupole mass spectrometer equipped with a TurboIonSpray electron spray ionization interface (PE Sciex, Canada) under multiple reaction monitoring conditions. The monitored MS/MS transitions of chitobiose and PNP-(GlcNAc)₃ were m/z 425([M+H]⁺) → 204 and m/z 749([M+H]⁺) → 204, respectively. The ratios between peak area of the sample product and those of the internal standard were determined, and the concentrations of samples were then calculated in relation to the internal standard. The concentration of each sample was measured in

duplicate and the mean was shown as the result. The detailed conditions of the HPLC-MS/MS for the quantitative analysis of chito oligosaccharides will be reported elsewhere.

RESULTS

Expression of chitinase genes in the presence of colloidal chitin

Chitinase production in *S. coelicolor* A3(2) was investigated in the presence or absence of colloidal chitin. Cells of *S. coelicolor* A3(2) M145 grown in LB were transferred to the inorganic salts medium with or without 0.05% (w/v) colloidal chitin, and chitinase activity in the culture supernatant was measured periodically using synthesized fluorescent substrates. Significant chitinase activity was detected 24 h after exposure to colloidal chitin whereas no activity was detected when cells were

cultured without colloidal chitin (data not shown). This indicates that chitinase production in *S. coelicolor* A3(2) was induced by colloidal chitin.

The transcription of the eight chitinase genes (*chiA*, *chiB*, *chiC*, *chiD*, *chiE*, *chiF*, *chiG* and *chiH*) in the presence or absence of colloidal chitin was then investigated periodically. Total RNAs used for Northern-blot hybridization were prepared from the cells grown with or without colloidal chitin after preculture in LB medium. In the presence of colloidal chitin, transcripts of 1.8, 2.0, 2.0, 1.4, 1.1 and 1.1 kb were detected with *chiA*, *chiB*, *chiC*, *chiD*, *chiF* and *chiG* probes, respectively, whereas no transcript was detected by either the *chiE* or *chiH* probe (Fig. 2), even when 10 times more RNAs (20 µg) were subjected to analysis (data not shown).

Fig. 2. Transcripts of chitinase genes in *S. coelicolor* A3(2) strain M145 in the presence or absence of colloidal chitin (CC). Northern-blot hybridization was performed using labelled anti-sense RNAs of *chiA* (a), *chiB* (b), *chiC* (c), *chiD* (d), *chiE* (e), *chiF* (f), *chiG* (g) and *chiH* (h) as probes. Three micrograms of total RNA was loaded per lane. The approximate sizes (kb) of the transcripts are indicated to the right of each panel. Cultures grown in LB were divided into two aliquots, and the mycelia in each aliquot were collected by centrifugation and resuspended in YE medium with or without 0.05% (w/v) colloidal chitin. Total RNAs were prepared from the mycelia grown in YE medium supplemented with colloidal chitin for 1, 2, 4, 6, 12 and 24 h, and from the mycelia cultured in YE medium for 0, 2, 6 and 24 h.

Fig. 3. (a) *chiC* transcripts in *S. coelicolor* A3(2) strain M145, and (b) calculated relative levels of the transcripts and the chitobiose concentration in the culture supernatant in the presence of colloidal chitin. Cultures grown in LB were resuspended in an inorganic salts medium supplemented with 0.05% (w/v) colloidal chitin at time zero and incubated. The mycelia were harvested every hour and total RNAs were extracted. The transcriptional products were detected by Northern-blot hybridization using the labelled anti-sense RNA of *chiC* as a probe. Two micrograms of total RNA was loaded on each lane. The approximate sizes (kb) of transcripts are indicated. The relative levels of the transcripts were measured by using a density-meter and are indicated by open triangles. Chitobiose concentration of the culture supernatant for each time was measured by HPLC-ESI-MS/MS and is shown by filled circles.

The *chiD* probe hybridized with a 2.0 kb extra band which was assumed to be the transcriptional product of the *chiC* gene (2.0 kb) as the *chiD* probe was highly homologous to *chiC* (81% at the DNA level) (Fig. 1). Furthermore, because the *chiG* and *chiF* genes share 86% similarity at the DNA level, the 1.1 kb band detected by the *chiG* probe was assumed to be the *chiF* transcript. The *chiC* probe hybridized with a 2.4 kb transcript in addition to the 2.0 kb major transcript. This larger transcript was presumed to be not those of *chiD* or *chiE*, since the 2.4 kb band was not detected by either the *chiD* or *chiE* probe. Considering that the *chiD* locus showed the highest similarity with *chiC* in the cosmid library representing the *S. coelicolor* A3(2) chromosome (Saito *et al.*, 1999), the 2.4 kb band was assumed to include the *chiC* transcript. From these results, we concluded that the transcription of *chiA*, *chiB*, *chiC*, *chiD* and *chiF* was induced by colloidal chitin. The transcripts of the five *chi* genes all showed the same temporal pattern, i.e. they reached maximum at 4 h after the exposure of cells to colloidal chitin and

decreased thereafter (Fig. 2). The induced levels of the *chiB* transcripts were significantly lower than those of the other four chitinase genes.

Dynamic analysis of induced transcription of chitinase genes

In order to compare the promoter activities of the chitinase genes, the accumulation of their transcripts was analysed dynamically. Total RNAs prepared from mycelia sampled at 1 h intervals following exposure to colloidal chitin were subjected to Northern-blot analysis. The amount of the *chiC* transcripts increased exponentially from 2 to 4 h after the addition of colloidal chitin (Fig. 3). The temporal patterns of the *chiA*, *chiD* and *chiF* transcripts showed a similar exponential increase (data not shown). These data enabled us to estimate the doubling times of transcript accumulation, which were used to compare the promoter activities of the chitinase genes irrespective of the difference in labelling efficiency of probes used in Northern-blot analysis. The doubling times of the *chiA*, *chiC*, *chiD* and *chiF* mRNA accumulations were 25, 22, 49 and 51 min, respectively. These values indicated that the promoter activities of the *chiA* and *chiC* genes were induced about twice as much as those of the *chiD* and *chiF* genes. Very low levels of the *chiB* transcription were detected only at 4, 5 and 6 h and the doubling time could not be calculated.

Transcription of chitinase genes induced by chitobiose

Chitin is hydrolysed to chitobiose, the dimer of N-acetylglucosamine, by *Streptomyces* chitinases (Blaak *et al.*, 1993; Ohno *et al.*, 1996). To determine whether the expression of chitinase genes is induced by chitobiose in *S. coelicolor* A3(2), chitinase activity in the culture supernatant and transcription of the eight *chi* genes were monitored. The mycelia were cultivated in the inorganic salts medium with or without chitobiose after preculture in LB medium. Significant chitinase activity was detected 4 h after the addition of chitobiose (1 mM or 250 µM), while no activity was detected in the absence of the substrate, indicating that chitinase production was induced by chitobiose (data not shown). To establish whether chitobiose induces the transcription of the *chi* genes, total RNAs prepared from mycelia exposed for 30, 60 and 120 min to 1 mM chitobiose were subjected to Northern-blot analysis. The results indicated that *chiA*, *chiB*, *chiC*, *chiD* and *chiF* were transcribed in the presence of chitobiose, and that the transcript levels of the genes increased through time (Fig. 4a). The transcript levels of the *chiB* gene were significantly less than those of the other four genes. No transcripts of *chiE*, *chiG* and *chiH* were detected even in the presence of chitobiose.

The temporal pattern of induction of the *chiA* transcription in the presence of chitobiose was compared with that of colloidal chitin. RNAs were taken every 2 h after the addition of the substrates. The maximum transcript level was reached at 2 h after exposure to

Fig. 4. Temporal patterns of the *chiA* transcripts in the presence of chitobiose (a, b) or colloidal chitin (c) in *S. coelicolor* A3(2) strain M145. Cultures grown in LB were divided into two aliquots, and the mycelia in each aliquot were harvested by centrifuging and resuspended in an inorganic salts medium supplemented with 1 mM (a) or 250 μM chitobiose (b), or 0.05% (w/v) colloidal chitin (c). Total RNAs were prepared from the mycelia grown in each medium for 30, 60 and 120 min (a) or 2, 4, 6, 9, 12 and 24 h (b, c). The transcripts were detected by Northern-blot hybridization using the labelled anti-sense RNA of *chiA* as a probe. Two micrograms of total RNA was loaded on each lane. The approximate sizes (kb) of transcriptional products are indicated.

250 μM chitobiose and at 4 h after the addition of colloidal chitin (Fig. 4b, c). This result for chitobiose, combined with the continuous increase of the transcript levels for 2 h after exposure to 1 mM chitobiose (Fig. 4a), suggests that *chiA* transcription reached the maximum level at 2 h following exposure to chitobiose. It was thus evident that chitobiose induced the *chiA* transcription significantly earlier than colloidal chitin. The same results were obtained for *chiB*, *chiC*, *chiD* and *chiF* (data not shown). The experiments were carried out twice and similar results were obtained. These results indicate that chitobiose has a more immediate effect on the transcriptional induction of the chitinase genes than colloidal chitin.

Chitobiose production from colloidal chitin in the culture supernatant

Because of the immediate effect of chitobiose on the transcriptional induction of the chitinase genes in *S. coelicolor* A3(2) (Fig. 4) and in *S. lividans* (Miyashita *et al.*, 2000), it was postulated that chitobiose is a direct inducer of chitinase gene transcription in these bacteria. If this is the case, chitobiose production should occur before the induced transcription of chitinase genes starts. To test this, samples were taken every hour from the inorganic salts medium culture supplemented with a 0.05% (w/v) colloidal chitin. After centrifugation, chitobiose concentration in the supernatant was determined, and the transcripts of the five *chi* genes in *S. coelicolor* A3(2) were measured using the total RNAs from the mycelia harvested from the corresponding cultures.

HPLC-tandem mass spectrometry enabled us to measure chitobiose concentration with high sensitivity. The chitobiose concentration increased exponentially from 0.06 μM (zero time) to 8.4 μM (4 h later) and further increased to 13.9 μM at 5 h (Fig. 3b). The experiment was carried out twice and similar results were obtained. The *chiC* transcripts were detectable at 2 h after exposure to colloidal chitin, when the chitobiose concentration in the culture supernatant was 0.66 μM. The levels of the *chiC* transcripts increased exponentially until 4 h and reached maximum at 5 h (Fig. 3). The increase of the *chiC* transcripts from 2 to 4 h after the addition of colloidal chitin seemed to coincide with the increase of the chitobiose concentration in the culture supernatant. These results, together with the more immediate effect of chitobiose than colloidal chitin on the induction of *chi* genes transcription (Fig. 4), suggest that chitobiose produced from colloidal chitin could be involved in the induction of the expression of chitinase genes in *S. coelicolor* A3(2).

Transcriptional induction of chitinase genes by α-chitin

Chitinase production in *S. plicatus* was shown to be induced by insoluble chitin but not by chitobiose (Robbins *et al.*, 1992). Crab shell chitin flakes retain the structure of α-chitin, unlike colloidal chitin, and are insoluble. To determine whether *chiE*, *chiG* and *chiH* are induced by chitin flakes, *S. coelicolor* A3(2) was grown in the presence of this substrate. Transcripts of the *chiA* gene were detected whereas those of *chiE*, *chiG* and *chiH* were not (data not shown).

Determination of the transcription start sites of *chiB*, *chiD* and *chiF*

In order to identify the promoter regions of the *S. coelicolor* A3(2) chitinase genes, the transcriptional initiation sites of the genes were deduced or determined. The promoter regions of the *chiA* and *chiC* genes of *S. coelicolor* A3(2) were deduced from the transcriptional initiation sites of the *chiA* and *chiC* genes of *S. lividans*

Fig. 5. Primer extension analysis to determine the transcriptional start points of *chiB* (a), *chiD* (b) and *chiF* (c). Lanes 1 through 4 are A-, C-, G- and T-specific dideoxy sequencing reactions, respectively. Lane 5 is the primer extension product. The corresponding sequences of the coding strands are shown on the right. The nucleotides of transcriptional starts, and the -10 and -35 regions of the promoter consensus sequences are indicated.

(Fujii & Miyashita, 1993; Miyashita & Fujii, 1993), respectively, because the corresponding genes in the two species are almost identical in their upstream regions and coding regions (Saito *et al.*, 1999). The deduced transcriptional initiation sites of *chiA* and *chiC* were the G and the ACT nucleotide residues at positions 51 and from 56 to 58 upstream of the putative translation initiation sites, respectively. To identify the promoter regions of *chiB*, *chiD* and *chiF*, their transcription initiation sites were determined by primer extension analysis using total RNAs prepared from cells grown for 2 h in an inorganic salts medium containing 250 μ M chitobiose. For *chiB*, the extension product corresponded to the G residue 39 nt upstream of the putative translation initiation site (Fig. 5a). For *chiD* and *chiF*, the extension products matched with the ACCG and CC nucleotide residues at positions from 66 to 69 and 119 to 120 upstream of the putative translation initiation site, respectively (Fig. 5b, c). The possible RNA polymerase binding sites of *chiE*, *chiG* and *chiH*, whose transcripts

were not detected by Northern-blot analysis, were searched using the SDC Genetix System (Software Kaihatsu Co.). A possible promoter sequence was found upstream of *chiE* (Fig. 6) whereas there was no obvious promoter sequence upstream of *chiG* and *chiH*.

Ni & Westpheling (1997) proposed that the direct repeat sequences in the *chi63* promoter region of *S. plicatus* direct its chitin-induced transcription. In *S. coelicolor* A3(2), sequences highly similar to the *S. plicatus* direct repeat are present in the upstream regions of *chiA*, *chiB*, *chiC*, *chiD*, *chiE*, *chiF* and *chiH* but not of *chiG*, while only one copy of the repeated sequence is present in the possible promoter region of *chiE* (Fig. 6).

Glucose repression of transcription of chitinase genes

To investigate the transcriptional repression of the five *chi* genes by glucose in *S. coelicolor* A3(2) and the involvement of *glkA* in this phenomenon, Northern-blot

P-chiA gccgccaactTGGTCCGTACCTacctcttgacaccttcaatTGGTCTGGACCaccttgtacgtcagcgg
P-chiB cgaggcacTGGTGAGGTGCGcaaggggttgacaacagaatTGGTCTGGACCaaattgggcgccctccac
P-chiC cccgggcgccacatTGGTCCAGACCTattgacctatTGGTCCAGACCTttctattctcgccgacttgcgg
P-chiD ttcggaacccaagaatTGGTCCGAACCAAttgaccacacTGGTCTAGTCCcttacgggtgacggaccgagc
P-chiF gaacgcaagttgaacTGGTCTACACCCTtgacTGGTACAGACCAaagcggttgagtatgcctccacacc
P-chiE agtgcgcgggcaagccccgcagcgacgggttgacgcacTGGTCCAGACCTtctacgttggggcgctcgac
P-chiG cggcggtgacgggCGGTCCGAGCGgtccggggagCGGTCCGACCTcttcgaggggtgcacggccgct
P-chiH tggctccggcgccggcgatTGGTCTAGTCCcattcTGGTCCAGACCAAttgcgtcccccggtgctccgg

Consensus

TGGTC(C/T)(A/G)GACC(A/T)

Fig. 6. Nucleotide sequences of the promoter regions of chitinase genes of *S. coelicolor* A3(2). The -35 and -10 sequences of each promoter are double- and single-underlined, respectively. The transcriptional start sites of *chiB*, *chiD* and *chiF* determined in this study and those of *chiA* and *chiC* deduced from those of *S. lividans* (Fujii & Miyashita, 1993; Miyashita & Fujii, 1993) are boxed. Nucleotide sequences of direct repeats are printed in upper-case letters and those identical to the direct repeats of *chiC* are printed in white on black background.

Fig. 7. Transcription of the *chiC* gene in the presence or absence of glucose in *S. coelicolor* A3(2) strain M145 and its *glkA* mutant M480. Cultures grown in LB were divided into three aliquots, and the mycelia in each aliquot were suspended in YE medium, in YE medium supplemented with 0.05% (w/v) colloidal chitin, or in YE medium supplemented with 0.05% (w/v) colloidal chitin (CC) and 1.0% (w/v) glucose. Total RNAs were prepared from mycelia grown for 4 h in each medium. Conditions for Northern-blot hybridization are described in the legend of Fig. 4.

hybridizations were performed in the wild strain M145 and its spontaneous *glkA* mutant M480. In M145, *chiC* transcription was induced in the presence of colloidal chitin but repressed in the presence of both glucose and colloidal chitin whereas transcription occurred regardless of the presence of glucose in strain M480 (Fig. 7). The same results were obtained with *chiA*, *chiB*, *chiD* and *chiF* (data not shown). It thus appears that *glkA* is involved in the glucose repression of the five chitinase genes in *S. coelicolor* A3(2) strain M145.

DISCUSSION

In this study, the expression of eight chitinase genes in *S. coelicolor* A3(2) was analysed at the transcription level. Either colloidal chitin or chitobiose induced the transcription of *chiA*, *chiB*, *chiC*, *chiD* and *chiF* but not of *chiE*, *chiG* and *chiH*. The promoter activities of *chiA* and *chiC* were induced twice as much as those of *chiD* and *chiF*, while that of *chiB* was weak. The -35 hexamers of the promoter regions of the *chi* gene are more highly conserved (TTGACN) than the -10

hexamers [(T/c)AcNtT] (Fig. 6). No obvious -35 or -10 hexamers could be found upstream of *chiH* and *chiG*. The absence of a 'good' promoter region in *chiH* and *chiG* might be responsible for their lack of, or very low, expression.

The 12 bp direct repeat in the *chi63* promoter region of *S. plicatus* directs both induction and repression of the *chi63* promoter (Delic *et al.*, 1992; Ni & Westpheling, 1997). Two copies of the 12 bp putative operator sequence were found in the promoter regions of most of the *S. coelicolor* *chi* genes (Fig. 6). The proposed consensus for this putative operator is TGGTC(C/T)-(A/G)GACC(T/A). One of the repeats is between the -10 and -35 region and the other is located (from 1 to 7 bp) upstream of the -35 hexamer. The distance between the two repeats greatly varies from gene to gene: 4/5 bp in *chiH* and *chiF*, 9/10 bp in *chiC* and *chiD*, 19/20 bp for *chiA* and *chiB*. It is noteworthy that the more upstream repeat is absent from the *chiE* promoter region whereas the two repeats are very close to each other (4 bp) in the *chiH* promoter region. All the actively transcribed chitinase genes *chiA*, *B*, *C*, *D* and *F* share a similar organization with the two repeats on both sides (but at various distances) of the -35 hexamer. The sequence of the more upstream repeat of the *chiB* gene is the most divergent from the proposed consensus. This might account for the very poor expression of *chiB*.

Recently, Nguyen (1999) reported that Reg1, a regulatory protein for the amylase gene of *S. lividans* (Nguyen *et al.*, 1997), binds to the promoter regions of several genes, including the *chiA* gene of *S. lividans*, that are subject to glucose repression. It was suggested that common sequences in the promoter regions, which are different from the direct repeat mentioned above, are involved in the DNA-protein interactions. Although sequences highly similar to the common ones were not observed in all the promoter regions of the chitinase genes of *S. coelicolor* A3(2), the binding ability of Reg1 to the promoters and the involvement of the protein in the regulation of the chitinase genes need to be elucidated.

glkA, a gene encoding an ATP-dependent glucose kinase (Angell *et al.*, 1992), was shown to be involved in the glucose repression of the expression of the glycerol kinase, agarase and α -amylase genes in *Streptomyces* (Seno & Chater, 1983; Virolle & Bibb, 1988; Angell *et al.*, 1992). The *glkA* gene also appeared to be involved in the glucose repression of chitinase production in *S. lividans* (Saito *et al.*, 1998). In the current study, it appears that *glkA* is involved in the glucose repression of transcription of the five chitinase genes (*chiA*, *chiB*, *chiC*, *chiD* and *chiF*) in *S. coelicolor* A3(2) strain M145. On the other hand, it is reported that a mutation in the *glkA* gene had no effect on glucose repression of the *chi63* gene in an *S. coelicolor ccrA1* genetic background (Ingram & Westpheling, 1995). The *ccrA1* mutation might suppress the *glkA* mutation directly or indirectly.

When *S. coelicolor* A3(2) was exposed to colloidal chitin, the chitobiose concentration in the culture supernatant increased during the first hour from 0.06 to 0.22 μ M, although no *chi* gene transcript was detected during that period. We assume that low levels of chitinases constitutively produced initially digested colloidal chitin into chitobiose, which, upon accumulation, triggered the induction of *chi* gene transcription. Upon reaching the peak at 5 h of exposure to colloidal chitin, the transcripts of the five chitinase genes started to decrease (Fig. 2). Similar phenomena have been observed in various other systems (Baumann *et al.*, 1996; Schwarts *et al.*, 1998; Leveau *et al.*, 1999; Iyer *et al.*, 1999). For instance, in 2,4-dichlorophenoxyacetate (2,4-D)-utilizing *Ralstonia eutropha* JM134(pJP4), the 2,4-D-induced transcription from TfdR/S-regulated promoters decreased soon after the initial increase (Leveau *et al.*, 1999). In this system, 2,4-dichloromuconate, a pathway intermediate in 2,4-D utilization, is the signal for TfdR/S-mediated expression. It was assumed that the induction of intracellular enzymes that degrade the signal molecule caused the decrease in the *tfd* gene expression (Leveau *et al.*, 1999). Although the signal molecule(s) for the induction of the chitinase genes in *S. coelicolor* A3(2) is still not identified, the other enzymes of the metabolic pathway for chitin or chitobiose utilization might degrade the signal molecule(s) or its precursor(s), resulting in the decrease of chitinase genes transcripts.

The five chitinase genes (*chiA*, *B*, *C*, *D* and *F*) were expressed simultaneously in the presence of colloidal chitin, although the induction levels of *chiB* were by far the lowest. This suggests that the five different chitinases work synergistically to degrade chitin, which is a heterogeneous polysaccharide. The importance of the multiplicity of the *S. coelicolor* A3(2) chitinases in chitin degradation could be elucidated by the enzymic characterization of the gene products.

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