RESEARCH ARTICLE

High expression levels of chitinase genes in *Streptomyces coelicolor* A3(2) grown in soil

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Abstract

Although *Streptomyces* species are major chitin-degraders in soil ecosystems, the expression of the diverse chitinase genes within *Streptomyces coelicolor* grown in soil has not been assessed. As a first step, the induction pattern of nine chitinase genes in *S. coelicolor* growing in autoclaved soil was compared with those in liquid cultures. The relative expression levels of nine chitinase genes were measured using real-time reverse transcription PCR. The expression of all chitinase genes was induced by chitin in both autoclaved soil and liquid cultures, but to different levels. The expression levels of five chitinase genes in autoclaved soil were significantly higher than those in the liquid cultures. In particular, a putative chitinase gene, chitinase H, showed the highest induction in autoclaved soil. The same induction pattern was confirmed in nonautoclaved soil, indicating that soil contains some factors affecting the expression of chitinase genes. The *chiH* gene product, ChiH, cloned in *Streptomycetes lividans* was secreted and exhibited chitin degradation activity that was stable within a wide range of acidic pHs. The disruption of *dasR*, a transcriptional regulator for the uptake of *N*-acetylglucosamine, abolished the expression of *chiH*, demonstrating that DasR is required for the regulation of ChiH expression.

Introduction

Soil microorganisms play important roles in the carbon, nitrogen, phosphorus and sulfur cycles in soil through the decomposition of organic materials derived from plants, animals and microorganisms such as cellulose and chitin (Stevenson & Cole, 1999). Chitin, a polymer of β-1,4-*N*-acetylglucosamine, is the second most abundant biomaterial in nature following cellulose. It has been estimated that the annual formation rate of chitin is 10^{10}–10^{11} tons (Gooday, 1990). Accordingly, chitinase (EC 3.2.1.14), which hydrolyzes the β-1,4-glucosidic bonds of chitin, is one of the most important enzymes involved in the maintenance of nutrient cycles in nature. Chitinase has also attracted attention as a biocontrol factor against plant pathogenic fungi because it can degrade fungal cell walls (Singh *et al.*, 1999). A wide variety of organisms including fungi, plants, insects and bacteria have been shown to produce various types of chitinases (Huynh *et al.*, 1992; Royer *et al.*, 2002; Adams, 2004). So far, a number of genes coding for chitinase enzymes have been identified and purified from numerous bacterial sources, for example *Bacillus circulans* (Watanabe *et al.*, 1990), *Serratia marcescens* (Suzuki *et al.*, 2002), *Streptomyces plicatus* (Robbins *et al.*, 1988), *Streptomycetes lividans* (Miyashita *et al.*, 1991), *Streptomyces thermoviolaceus* (Tsujibo *et al.*, 1993) and *Streptomyces coelicolor* (Saito *et al.*, 1999). In particular, streptomycetes are well known to be decomposers of chitin in soil (Metcalfe *et al.*, 2002), and possess diverse and multiple chitinase genes with different enzyme characteristics (Romaguera *et al.*, 1992; Saito *et al.*, 1999).

Streptomyces chitinases are classified into two families of glycoside-hydrolases based on their amino acid sequences and the similarity of their catalytic domains, i.e. family 18 (consisting of three subfamilies, A, B and C) and family 19 (CAZyDB at http://cazy.org/Glycoside-Hydrolases.html) (Henrissat, 1991; Kawase *et al.*, 2006). Multiple chitinase genes of each family are commonly found in the genomes of

many streptomycetes (Saito *et al.*, 2003). Streptomycete chitinases play important roles cooperatively in the biodegradation of chitin in the soil ecosystem as well as in the protection of plants against pathogenic fungi (Watanabe *et al.*, 1999; Gomes *et al.*, 2000). In fact, it has been reported that there is a significant increase in the population of streptomycetes in soil after the addition of chitin or fungal cell walls (Williams & Robinson, 1981; Gomes *et al.*, 2000).

The mechanisms for the regulation of chitinase production in streptomycetes have primarily been investigated using liquid cultures. Chitinase production by streptomycetes is induced by chitin and repressed in the presence of readily utilizable carbon sources such as glucose (Miyashita *et al.*, 2000). The regulation of chitinase production by streptomycetes occurs at the level of transcription, and a pair of conserved direct repeat sequences has been found to play important roles in chitinase gene regulation (Delic *et al.*, 1992; Ni & Westpheling, 1997). The conserved sequences have been found not only in the promoter regions of chitinase genes but also in the genes of the sugar phosphotransferase system (PTS) for the uptake of *N*-acetylglucosamine (Rigali *et al.*, 2004, 2008). The PTS genes have been shown to be regulated by a GntR-like transcriptional regulator, DasR, a member of a class of transcriptional regulators that typically respond to metabolite effector molecules (Rigali *et al.*, 2006; Hoskisson & Rigali 2009), and that DasR bound to the conserved sequences of chitinase promoters (Colson *et al.*, 2007). Therefore, they were designated as DasR-responsive elements (*dre*) (Colson *et al.*, 2007).

Streptomyces coelicolor A3(2) is a well-studied streptomycete strain whose entire genome sequence is known (ScoDB at http://strepdb.streptomyces.org.uk), and that possesses more than ten chitinase genes (some of which are putative). Moreover, the induction mechanisms of chitinase gene expression have been studied well in liquid cultures (Saito *et al.*, 2000; Colson *et al.*, 2007). Thus, this strain is an ideal model strain for studying the actual roles of chitinases in the biodegradation of chitin in nature or their antifungal activities in the soil environment. Despite decades of research on *S. coelicolor* chitinases, however, the mechanism of chitinase induction and the chitinase expression profiles in cells growing in soil are unclear, because it is technically quite difficult to obtain sufficient yields of pure RNA from soil. RNA extraction is a challenge not only because of the heterogeneity of soil but also due to the instability of mRNA. Moreover, the presence of humic substances and indigenous materials in soil can interfere with the RNA extraction procedure and postextraction of the enzymatic reaction (Saleh-lekha *et al.*, 2005). In this study, we constructed a model soil culture system of the *S. coelicolor* A3(2) strain using nonautoclaved and autoclaved soil, and compared the expression patterns of chitinase genes in soil and liquid cultures. Significant upregulation of a putative chitinase gene, *chiH*, in soil was observed. Moreover, the expression of *chiH* was found to be completely dependent on *dasR*. The role of the DasR regulator in the regulation of chitinase genes is discussed.

Materials and methods

Bacterial strains, plasmids, media and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. Brown forest soil obtained from a field at the Ehime Agricultural Experiment Station, Japan (FAO classification, Gleyic Cambisols), was used in all experiments. The preparation of soil samples and the soil properties have been reported previously (Wang *et al.*, 2008). The nonautoclaved and autoclaved soil samples were adjusted to 60% of the maximum water-holding capacity with sterilized water (as a control) or a sterilized solution of colloidal chitin to yield a final 0.2% (w/w) of colloidal chitin (for the induction of chitinase genes), and then distributed in 2 g portions into 15-mL sterile disposable plastic tubes. Spores of *S. coelicolor* A3(2) or its *dasR*-disrupted mutant, YU1 (final 10^6 CFU g^{-1} soil for each), or wet mycelia of *S. coelicolor* A3(2) (1 mg g^{-1} soil) were inoculated into the soil, followed by incubation at 30 °C for various durations of time. Colloidal chitin was prepared using the method described previously (Lingappa & Lockwood, 1962). To monitor the population of *S. coelicolor*, total DNA was extracted from the soil at various times during growth as described previously (Morimoto *et al.*, 2008).

Inorganic salts liquid medium containing 0.2% (w/v) colloidal chitin (Miyashita *et al.*, 1991) was used to cultivate *S. coelicolor* A3(2) and its *dasR*-disrupted mutant, YU1. Briefly, a spore stock was added to 100 mL of liquid medium in a 500-mL baffled Erlenmeyer flask to yield a final concentration of 10^6 CFU mL^{-1}, after which the culture was incubated at 30 °C with shaking at 200 r.p.m. The spore stock of each strain as well as SFM agar medium, yeast extract malt extract (YEME) medium and R2YE agar medium were prepared as described previously (Kieser *et al.*, 2000). YEME was used for the overproduction of the gene product of *chiH* in *S. lividans* TK24 (Kieser *et al.*, 2000). Luria–Bertani (LB) medium was used to prepare mycelia as the first culture for inoculation of a soil culture as described previously (Saito *et al.*, 2000).

RNA preparation

To determine the time course of the expression of each gene, RNA was prepared at 24, 48, 60 and 72 h after inoculation with spores from soil with or without colloidal chitin and inorganic salts liquid cultures containing colloidal chitin. A nonautoclaved soil without spore inoculation supplemented

Table 1. Bacterial strains and plasmids vectors used in this study

Organisms and plasmids	Characteristics	Source or reference
Escherichia coli		
JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *e*14$^-$, *supE*44, *relA*1, Δ*lac-proAB*)/F′ [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Purchased from Takara, Japan
ET12567	Km^{R}Cm^R pUZ8002	Kieser *et al*. (2000)
S. coelicolor A3(2) M145	SCP1$^-$ SCP2$^-$ (parental strain)	Bentley *et al*. (2002)
S. lividans 66 TK24	SCP1$^-$ SCP2$^-$ (wide-type strain)	Hopwood *et al*. (1983)
Plasmid vectors		
pEMJ7	pIJ702 derivative containing the gene fragment of *chiC S. lividans*	Miyashita *et al*. (1991)
pGEM-T Easy	Cloning vector of the PCR product	Purchased from Promega
pIJ6021	Tsr^R, Kan^R (expression vector for *Streptomyces*)	Takano *et al*. (1995)
pIJ487	Tsr^R, Kan^R, Neo^R (promoter probe vector)	Ward *et al*. (1986)
pIJ2925	pUC18 derivative vector with BglII sites flanking modified multiple cloning sites	Janssen & Bibb (1993)
pAS100	pGM160 derivative of a temperature-sensitive plasmid, from which the HindIII fragment including the *acc*C4 gene has been removed	Xiao *et al*. (2002)
pDR04	pAS100 derivative, in which the BamHI/XbaI fragment containing the hyg gene, and upstream and downstream of the *dasR* gene has been replaced	This study
pCDR24	pSET152 derivative, containing the wild-type *dasR gene* fragment and the apramycin-resistant gene	This study
pEM-H1	pIJ6021 derivative containing the structural gene of *chiH*	This study
pEM-H2	pIJ487 derivative containing the chiH gene with its own promoter	This study

with 0.2% (w/w) of colloidal chitin was used as a control. Total RNA was extracted from 2 g soil samples using an RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. To obtain enough RNA (at least 2 μg) from soil samples for real-time RT-PCR analysis, the RNA samples extracted at each of the sampling points were mixed, purified using a MicroSpin S-400 HR spin column (GE Healthcare, Little Chalfont, UK), subjected to DNase digestion using a TURBO DNA-Free Kit (Ambion, Austin, TX) and then concentrated using an RNA Clean-up & Concentration Kit column (Zymo Research, Orange, CA) as described previously (Wang *et al*., 2009). At each of the sampling points for the liquid cultures, 10 mL of culture was removed and centrifuged for 10 min at 890 ***g***. RNA extraction from the obtained pellets and purification of the RNA samples were subsequently performed using an RNeasy Mini Kit (Qiagen, Valencia, CA) and a TURBO DNA-Free Kit (Ambion), respectively, according to the manufacturer's instructions. The concentrations of RNA were determined using a Nanodrop system (Nanodrop, Wilmington, DE). Three independent RNA samples were extracted from soil and liquid cultures, as biological replicates, for real-time RT-PCR analyses.

Real-time RT-PCR

The expression of chitinase genes and *hrdB* was examined using two-step real-time RT-PCR. The specific primers used for real-time RT-PCR are listed in Table 2. Total RNA extracted from soil and liquid cultures (2 μg) was reverse transcribed using a PrimeScript^{TM} RT Reagent Kit for RT-PCR (Takara, Shiga, Japan) according to the manufacturer's instructions for random hexamer-primed reactions with the following modification: RNA was preheated at 70 °C for 5 min and then cooled on ice for 1 min before the addition of the reaction mixture. Real-time PCR was conducted using an SYBR Premix DimerEraser (Perfect Real Time; Takara) on a StepOnePlus Real-Time PCR Detection System (Applied Biosystems, Foster City, CA). One microliter of the above reaction mixture containing synthesized cDNA was used as a template in a 20 μL volume of the SYBR PremixDimerEraser reaction mixture containing 300 nM of each primer. The reaction conditions were as follows: one cycle at 95 °C for 10 s to activate the DNA polymerase, followed by 40 cycles of 10 s at 95 °C, 30 s at 59 °C (60 °C for chiM) and 32 s at 72 °C, and then a melting curve stage, which generated melting curves with continuous fluorescence acquisition from 60 to 95 °C at a rate of 0.3 °C s^{-1}. Standards for the assays were prepared with PCR amplicons from the total DNA of *S. coelicolor* A3(2) using the same primers as those used for real-time PCR. A standard curve was constructed by comparing the copy numbers of 10-fold dilutions of the standard with the respective threshold cycles. All reactions were performed in triplicate. The *hrdB* gene encoding a major sigma factor was used as an inner control to quantify the relative expression of chitinase genes and water as a negative control (Roth *et al*., 2004; Alduina *et al*., 2007).

Table 2. Primers used in this study

Primer name	Sequence	Description	Product size (bp)
chiA-F	ACGGTCTCAACCCGACGTA	Used to quantify SCO5003(*chiA*) transcripts	137
chiA -R	CAGCGCGGTCTGGAAGTAG		
chiC-F	GGTGAAGCTGTCCTGGTC	Used to quantify SCO5376(*chiC*) transcripts	117
chiC-R	AGGCCGTTGTCCGTGTAG		
chiD-F	CACGTACGAGAACGGCATC	Used to quantify SCO1429(*chiD*) transcripts	117
chiD-R	GTGTCGTAACTCCACCAGTC		
chiE-F	AGCAGCTCAAGGTCGGGT	Used to quantify SCO5954*chiE* transcripts	130
chiE-R	GGGTCGAGGTTCTCGAAG		
chiF-F	CACAACGCGATGGTCAAC	Used to quantify SCO7263*chiF* transcripts	111
chiF-R	CTTGGTGACGCGGCTCTG		
chiH-F	CATCACGACCTACGACGTCTC	Used to quantify SCO6012*chiH* transcripts	100
chiH-R	CAGCTTGATGGCCTTGTTG		
chiI-F	GGTCATCCACAGCGACCA	Used to quantify SCO1444 *chiI* transcripts	132
chiI-R	TAGTAGTTGAACGCCAGGTCCAC		
chiJ-F	TCCCAGTCCAACCTGATCC	Used to quantify SCO2503*chiJ* transcripts	124
chiJ-R	AGATCGATCCGTAGACGATG		
chiM-F	CGTGAGCCACCAGGTGAA	Used to quantify SCO7225*chiM* transcripts	108
chiM-R	TTGACGTCGATGCAGGAGTAG		
hrdB-F	TCGACTACACCAAGGGCTACAA	Used to quantify SCO5820*hrdB* transcripts	115
hrdB-R	ACCATGTGCACCGGGATAC		
dasR-F	TTCGTCGCCAAGCCCAAGGT	Used to quantify SCO5231*dasR* transcripts	124
dasR-R	GGTCGTCGGCGGTGATGTAGC		
ChitH2 F	GACTcatATGAGACGCATGCGTTCCATCCG	The NdeI site is attached (underlined)	1524
ChitH2 R	GTGggaTCCTCAGCAGGCGCCGAGGTCCTG	BamHI site is attached (underlined)	
P-ChitH F	GGGgaaTTCTACGTCTTCCCGGTC	Used to amplify *chiH* with its promoter region. ChitH2R used as a reverse primer. The EcoRI site is attached (underlined)	1784
CbiRUf	tctagaTTCTTGTTGTTGTAGACGACGAC	Used to amplify the *dasR* upstream region	975
CbiRUr	ggatccTCGTTCTCCGCACTGCTGAC	The XbaI or the BamHI site is attached (underlined)	
CbiRDf	ggatccGCGACCGCTACAGTTCGTG	Used to amplify the *dasR* downstream region	1018
CbiRDr	gaattcGTCCCGACGAGTACGTGTAC	BamHI or EcoRI site is attached (underlined)	
E4UPr	GGCTATAAGCTTGCGCTTCAC	Used to amplify the *dasR* and its promoter region. CbiRDr used as a reverse primer.	2206

Sequences corresponding to the native sequences are shown in upper case, whereas artificially attached nucleotides are indicated in lower case.

Disruption of *dasR*

A *dasR* mutant, YU1 (M145 *dasR*::*hyg*), was constructed by replacing nucleotides 33–738 by a hygromycin cassette (Blondelet-Rouault *et al*., 1997) with an orientation that was opposite to that of the residual *dasR* gene using a temperature-sensitive vector, pAS100 (Xiao *et al*., 2002). A *dasR*-disruption plasmid, pDR04, harboring PCR-amplified DNA fragments containing regions (~1 kb) upstream and downstream of the *dasR* gene (SCO5231), separated by the *hyg* cassette, was constructed. *Streptomyces coelicolor* A3(2) was transformed with pDR04 prepared from *Escherichia coli* ET12567 according to the method described by Kieser *et al*., 2000. After obtaining thiostrepton-resistant transformants at 30 °C, we selected hygromycin B-resistant, but thiostrepton-sensitive strains that lost the plasmid after incubation at 39 °C on SFM agar medium. Disruption of *dasR* was verified by Southern blot analysis using the labeled *dasR* and *hyg* genes as probes. For the complementation of the *dasR* mutation of YU1, a derivative of an insertional plasmid, pSET152 (Bierman *et al*., 1992), harboring the wild-type *dasR* gene and its promoter region encompassing nucleotide positions −476/+1724 relative to the start of *dasR* was introduced into YU1. The apramycin-resistant transformant obtained was designated as YU1MR. The insertion of the wild-type *dasR* gene fragment into the genome was confirmed by Southern blot analysis using the labeled *dasR*.

Cloning and purification of a gene product, *chiH*

A PCR-amplified DNA fragment containing the coding region of the *chiH* gene was inserted at the NdeI–BamHI sites of pIJ6021 (Takano *et al*., 1995) to yield pEM-H1, which was then transformed into *S. lividans* TK24. In the transformants, the *chiH* structural gene was under the

control of the *tipA* promoter that was induced upon the addition of thiostrepton (Takano *et al.*, 1995).

To confirm the regulation of *chiH* gene expression by its own 'promoter', an EcoRI–BamHI fragment containing the *chiH* gene with 248 bp upstream of the structural region of *chiH* was inserted into the corresponding sites of pIJ487 (Ward *et al.*, 1986) to yield pEM-H2, which was subsequently cloned in *S. lividans* TK24.

Overproduction of ChiH was induced by the addition of thiostrepton (final concentration, 5 μg mL^{-1}) at 30 °C overnight in 100 mL of YEME medium. Proteins in the obtained culture supernatant were precipitated with 80% ammonium sulfate. The precipitate was suspended in 10 mL of 10 mM potassium phosphate buffer, pH 7.2, dialyzed against the same buffer, and then applied to a HiPrep DEAE FF column (16/10 cm) (GE Healthcare) equilibrated with the same buffer. The proteins bound to the column were eluted by increasing the ionic strength of KCl as a linear gradient from 0.01 to 1 M using an ÄKTA system (GE Healthcare). The homogeneity of the purified ChiH protein was confirmed by 10% SDS-PAGE.

Characterization of ChiH

The N-terminal amino acid sequence of the purified ChiH protein was determined by GenoStaff Co. Ltd (Tokyo, Japan), using the purified ChiH band identified upon SDS-PAGE analysis. For the determination of the molecular mass of the purified enzyme, the purified ChiH was applied to a Superdex G200 (10/300) column (GE Healthcare),which was then attached to an ÄKTA purifier, followed by elution with 50 mM KPB (pH 7.2) containing 0.15 M KCl at a flow rate of 0.5 mL min^{-1}. The $A_{280\,nm}$ of the effluent was recorded. The molecular weight of the enzyme was calculated by comparison with the mobilities of the following standard proteins: catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and RNase A (13.7 kDa). Chitinase activities were measured using a fluorescent substrate, 4-methylumbellifery-*N*,*N*′-diacetylchitobioside [4-MU-$(GlcNAc)_2$)], or 4-methylumbellifery-*N*,*N*′,*N*″-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] (Sigma), as described previously (Miyashita *et al.*, 1991). The optimum pH of ChiH was determined by measuring chitinase activities at pH values ranging from 2 to 10.5 at 37 °C. The effect of pH on the activity of ChiH was estimated by measuring the residual chitinase activities after incubation at 25 °C for 30 min at various pH values in the following buffers at a concentration of 0.5 M: Glycine-HCl buffer (pH 2.0–4.0), citrate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 7.5–9.0) and NH_4OH-NH_4Cl (pH 9.0–10.5).

Results

Growth of *S. coelicolor* A3(2) in autoclaved soil

To determine the gene expression pattern of chitinases in *S. coelicolor* A3(2) in the soil environment, as a first step, autoclaved soil was used to eliminate the background of transcripts of orthologues derived from resident streptomycetes. To monitor the population of *S. coelicolor* A3(2), total DNA was directly extracted from soil and the DNA copy number of a single copy gene for a principal sigma factor, *hrdB*, of *S. coelicolor* A3(2) was measured by real-time PCR (Fig. 1a and b). The increasing rate of the copy number of *hrdB* was almost identical to that of the amount of total DNA extracted from soil (Fig. 1c). At first, the growth patterns of *S. coelicolor* A3(2) in autoclaved soil obtained by inoculation with spores were compared with inoculation with mycelia prepared from an LB culture. Inoculation of mycelia into soil resulted in a significant decrease in the copy number of *hrdB* recovered from the soil at 120 h after

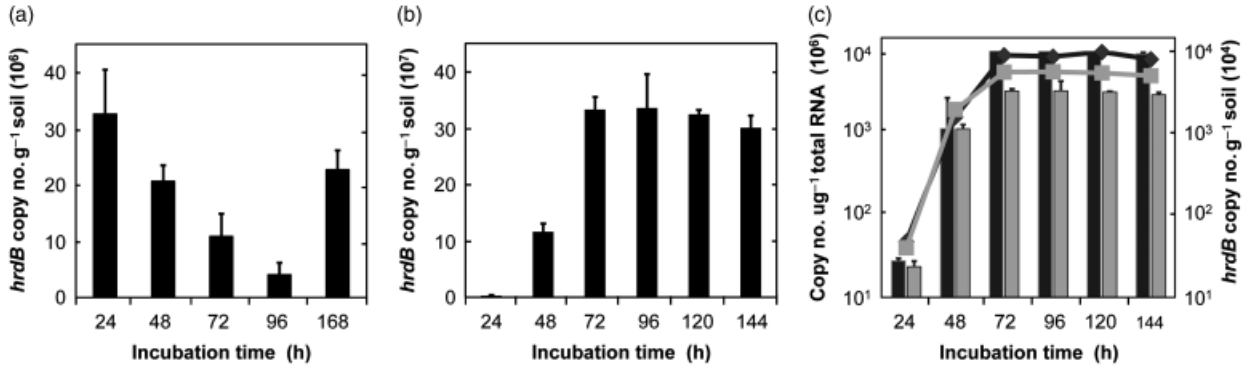

Fig. 1. Monitoring of the population of *Streptomyces coelicolor* A3(2) in autoclaved soil. The population of *S. coelicolor* A3(2) in soil inoculated with mycelia (a) or with spores (b) was monitored by the DNA copy number of the sigma factor *hrdB* determined by real-time PCR. The population of *S. coelicolor* A3(2) was monitored by the DNA amount extracted from soil (◆ or ■) or the *hrdB* copy number (black bars or gray bars) (c). The population was also compared in the presence (◆ and black bars) and absence (■ and gray bars) of colloidal chitin in autoclaved soil inoculated with spores of *S. coelicolor* A3(2).

inoculation, indicating lysis of the mycelia inoculated into the soil. Conversely, a rapid increase in the *hrdB* copy number was observed in the soil inoculated with spores, especially within 72 h after inoculation. Based on these data, we performed spore inoculation to prepare soil and liquid cultures for gene expression analysis.

Induction of chitinase gene expression in autoclaved soil

Streptomyces coelicolor A3(2) grown in autoclaved soil with and without colloidal chitin showed good growth, the growth rates being almost the same, until 72 h after spore inoculation (Fig. 1c). We prepared RNA samples from autoclaved soil at various times after spore inoculation and then determined the copy numbers of nine chitinase genes by real-time RT-PCR. We found that the existence of a substrate, colloidal chitin, was necessary for the transcriptional induction of chitinase genes in soil with different induction levels (Fig. 2a). Specifically, the induction levels of *chiA, C, H* and *I* were higher than those of *chiD, E, F, J* and *M*. For all chitinase genes, the samples prepared 48 h after spore inoculation showed the highest level of induction during growth in autoclaved soil. A putative chitinase gene, *chiH*, showed especially high expression among the chitinase genes. Without the substrate, a very low level of transcription was observed for each chitinase gene. This is the first report of the detection of the expression of *chiH, I, J and M* in *S. coelicolor* A3(2) to date.

Comparison of chitinase gene expression in autoclaved soil and liquid cultures

To determine whether the gene expression pattern in autoclaved soil was the same as that in a liquid culture, we prepared an inorganic salts liquid culture containing colloidal chitin and then the transcript copy number of each chitinase gene was determined at various times after spore inoculation by real-time RT-PCR. The *hrdB* gene was used as a control to quantify the relative expression of chitinase genes under different conditions, because the ratio of *hrdB* transcripts to total RNA was almost constant throughout the growth period in the autoclaved soil and liquid cultures (Fig. 3). We calculated the relative expression level of each

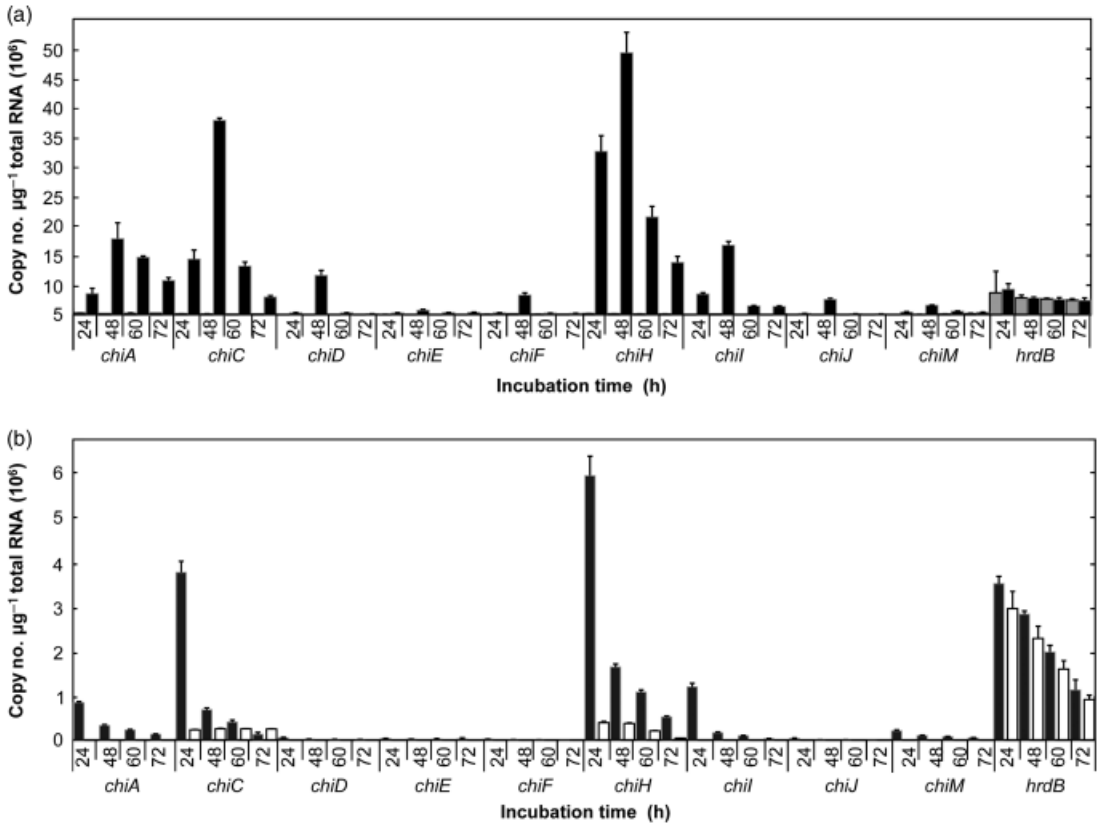

Fig. 2. Time course of the gene expression of chitinases and the major sigma factor, *hrdB*, in *Streptomyces coelicolor* A3(2) growing in autoclaved soil (a) or nonautoclaved soil (b). Transcriptional activity was evaluated based on the transcript copy number of each gene determined by real-time RT-PCR in the presence (■) or absence (▩) of colloidal chitin with inoculation of *S. coelicolor* (a). The gene expression of chitinases in nonautoclaved soil was measured in the presence of colloidal chitin with (■) or without (□) the inoculation of *S. coelicolor* (b).

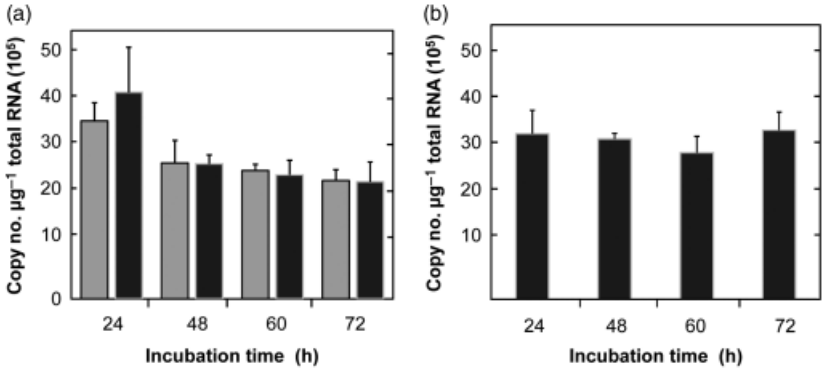

Fig. 3. Expression of *hrdB* during growth in various *Streptomyces coelicolor* A3(2) cultures. The expression of *hrdB* in autoclaved soil (a) and inorganic salts liquid cultures (b) was evaluated based on the transcript copy number determined by real-time RT-PCR in the presence (■) or absence (□) of colloidal chitin.

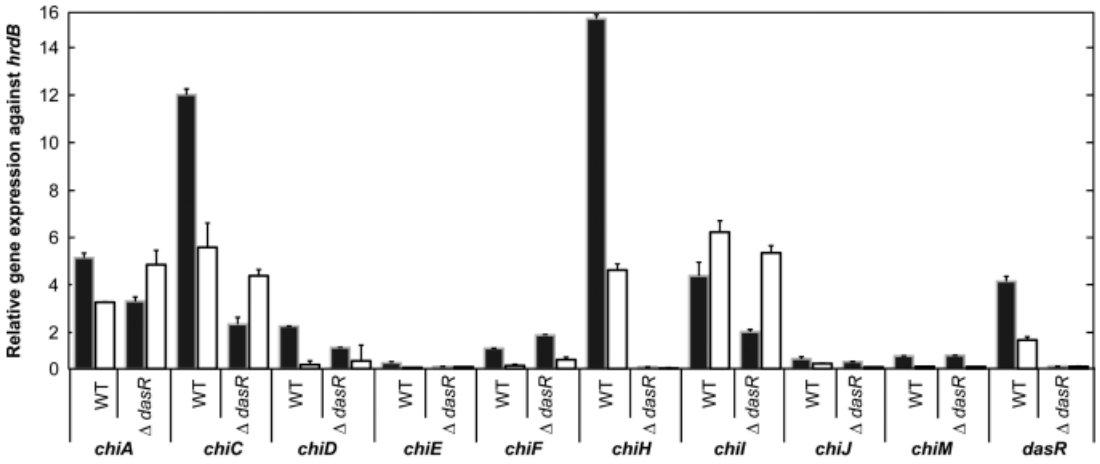

Fig. 4. Relative gene expression levels of chitinase genes in soil and liquid cultures. The wild type and *dasR*-null mutant (YU1) strains of *Streptomyces coelicolor* A3(2) were cultivated in soil (■) or inorganic salts medium (□). The time course of the expression level of each gene was monitored in each culture as in Fig. 1. The relative gene expression levels of chitinases and *dasR* against *hrdB* were calculated based on the transcript copy number determined by real-time RT-PCR. In the figure, only the maximum induction levels for each gene during the exponential growth phase are indicated.

chitinase gene against *hrdB* based on the copy numbers and compared the induction patterns of the chitinase genes in autoclaved soil with those in liquid cultures (Fig. 4). The analyses indicated that the relative induction levels of *chiA, C, D, F* and *H* in the autoclaved soil were significantly higher than those in the liquid cultures, while *chiI* showed a higher induction level in the liquid cultures. Moreover, the induction levels of *chiC* and *H* were especially high in the autoclaved soil, being 2.25- and 3.55-fold higher than that in the liquid cultures, respectively (Fig. 4).

Induction of chitinase gene expression in nonautoclaved soil

To determine whether the results obtained for autoclaved soil were due to the autoclaving or not, the gene expression patterns of chitinases of *S. coelicolor* A3(2) were measured in nonautoclaved soil containing colloidal chitin. It was found that the expression pattern showed a similar tendency as in the autoclaved soil, while the expression level of each chitinase was rather low (Fig. 2b). In particular, *chiC* and *H* were also highly induced in the nonautoclaved soil. Very low expression levels of chitinase genes were observed without inoculation of *S. coelicolor* A3(2), indicating that most of the chitinase transcripts observed were derived from the inoculated *S. coelicolor* A3(2).

The effect of *dasR* disruption on the induction of chitinase gene expression

Previous observations revealed that a GntR-family transcriptional regulator, DasR, binds to *dre*, which is located in the promoter regions of chitinase genes including *chiH* (Ni & Westpheling, 1997; Colson *et al.*, 2007). To clarify the role of DasR in the regulation of chitinase gene expression in soil and liquid cultures, we constructed a *dasR*-disrupted mutant of *S. coelicolor* A3(2), YU1, and then compared the gene expression of chitinases in the wild type and YU1 in

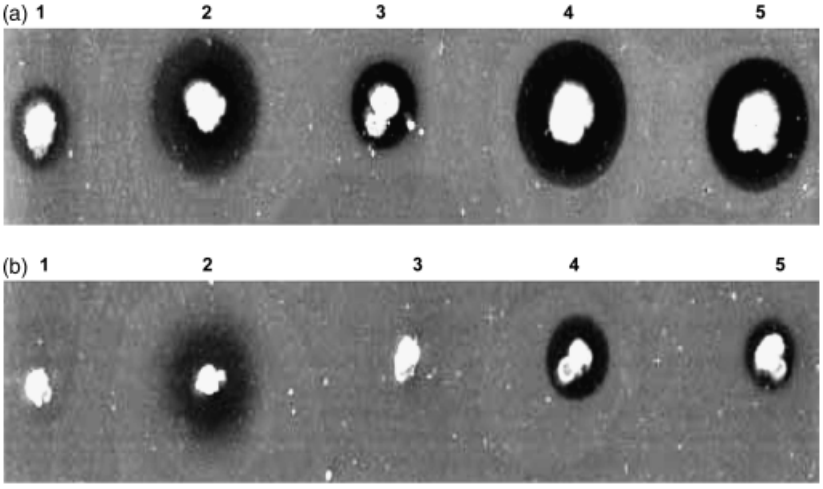

Fig. 5. Chitinase activity of *chiH* clones of *Streptomycetes lividans* TK24 on inorganic salts colloidal chitin agar plates. Spore suspensions of *S. lividans* TK24 harboring pIJ6021 (1), pEM-H1 (*chiH* under control of the *tipA* promoter) (2), pIJ487 (3), pEM-H2 (*chiH* with its own promoter) (4) and pEMJ7 (*chiC* with its own promoter) (5) were spotted onto an inorganic salts agar plate containing 0.15% of colloidal chitin plus 10 μg mL^{-1} thiostrepton (a) and an inorganic salts colloidal chitin agar plus 10 μg mL^{-1} thiostrepton plate containing 1% glucose (b). The chitinase activity of each strain is visualized as the formation of a halo around the colonies.

soil and liquid cultures. Real-time RT-PCR analysis showed that a *dasR* mutation had a different effect on the expression of each chitinase gene (Fig. 4). The most striking effect was observed in *chiH* expression. Specifically, the expression of *chiH* was completely attenuated in YU1 in both soil and inorganic salts liquid cultures, indicating that DasR is necessary for the induction of *chiH* expression (Fig. 4). A similar, but partial reduction of the gene expression level was generally observed for most chitinase genes in both soil and liquid cultures. However, the expression level of *chiA* in liquid cultures and that of *chiF* in soil and liquid cultures were higher in YU1 than in the wild type. The *dasR* mutant was complemented by the insertion of a plasmid harboring the wild-type *dasR* gene and its promoter region into its genome. In the complemented mutant, YU1MR, the induction of *chiH* was recovered in soil (Supporting information, Fig. S1).

Cloning of *chiH*

A putative chitinase gene, *chiH* (SCO6012), was identified by the *S. coelicolor* A3(2) genome sequencing project and classified into subfamily C of the family 18 chitinases (Kawase *et al.*, 2006). So far, neither the gene expression of *chiH* nor the enzyme activity of the gene product of *chiH*, ChiH, has been confirmed as yet experimentally. To determine whether ChiH has chitinase activity, the structural gene of *chiH* was cloned and expressed in *S. lividans* TK24 using expression vector pIJ6021. The obtained transformed TK24 strain harboring pEM-H1 formed larger clear zones than a control on inorganic salts agar plates containing colloidal chitin and 10 μg mL^{-1} thiostrepton, an inducer of the *tipA* promoter (Fig. 5). These findings indicate that the structural gene of *chiH* under control of the *tipA* promoter encodes an actual chitinase enzyme that can be secreted outside of cells. The recombinant containing a pIJ487 derivative, pEM-H2, with a *chiH* gene possessing its own putative promoter (the structural gene of *chiH* and its 248 bp upstream region) also produced a large clear zone on colloidal chitin inorganic salts agar plates (Fig. 5), indicating that colloidal chitin induces a high level of expression of ChiH from its own promoter. Clear zone formation around the colony of TK24 harboring pEM-H2 was inhibited in the presence of 10 mg mL^{-1} glucose, demonstrating that the production of ChiH was under the control of catabolite repression, similar to that of ChiC (Fig. 5). The regulation of ChiC has already been investigated in detail (Miyashita *et al.*, 1991; Fujii & Miyashita, 1993). It is induced in the presence of chitobiose and colloidal chitin, and repressed by glucose.

Purification and characterization of ChiH

Overproduction of ChiH in a culture of *S. lividans* TK24 harboring pEM-H1 was induced by thiostrepton, and then the ChiH protein was purified. The N-terminal amino acid sequence of the purified ChiH protein was found in the deduced amino acid sequence of ChiH in a database (T_{35}PLPD$_{39}$). The molecular mass of the purified ChiH was estimated to be 49 kDa by gel filtration column chromatography and SDS-PAGE (Fig. S2), which was largely consistent with that of the deduced amino acid sequences of the secreted form of ChiH from T_{35} to C_{507} (473 aa, MW = 49 726.67 Da). The purified ChiH released 4 MU from 4-MU-$(GlcNAc)_3$ more rapidly than from 4-MU-$(GlcNAc)_2$ (Table 3). The optimum pH for the enzyme activity of ChiH was pH 5.2 (Fig. 6a). The purified ChiH was stable in a wide acidic pH range (2.0–6.0) (Fig. 6b).

Discussion

For gene expression studies, it is crucial to obtain RNA samples from actively growing cells (Wang *et al.*, 2008).

Table 3. Purification of ChiH from *Streptomyces lividans* TK24 in YEME medium

		Chitinase activity (4 MU-trichitobioside)			Chitinase activity (4 MU-dichitobioside)		
Step	Total protein (mg)	Sp. act. (U mg^{-1})	Total *U* (A)	Yield (%)	Sp. act. (U mg^{-1})	Total *U* (B)	Ratio (B/A)
Culture supernatant	32.32	15.7	506.1	100	10.4	337.8	0.67
$(NH_4)_2SO_4$ ppt (80%)	18.60	25.5	474.3	93.7	15.7	292.7	0.62
HiPrep DEAE FF	0.53	308.9	163.7	32.3	179.6	95.2	0.58

ppt, precipitate; sp. act., specific activity.

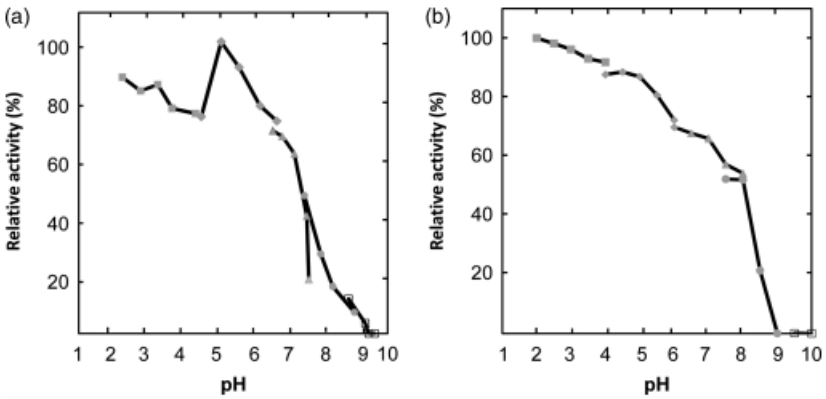

Fig. 6. Optimum pH and pH stability of ChiH. To determine the optimum pH (a), reactions were conducted for 2 min at 37 °C in the following buffers (0.1 M): Glycine-HCl buffer (■), citrate buffer (◆), potassium phosphate (▲), Tris/HCl buffer (●) and NH_4OH-NH_4Cl (□). The pH stability of ChiH (b) was determined by measuring the residual chitinase activity after incubation for 30 min at the various pHs of the above buffers. Relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions used.

Therefore, in the present study, we determined the conditions under which *S. coelicolor* could grow actively in soil. For monitoring the population of *S. coelicolor*, it is not suitable to determine CFUs using conventional plating methods, because *S. coelicolor* shows filamentous growth. It was impossible to distinguish whether the grown colonies were derived from inoculated spores or growing mycelia on agar plates. As indicated in Fig. 1c, the DNA amount or the copy number of the *hrdB* gene recovered from soil increased significantly within 72 h after inoculation of spores. We decided to use the DNA copy number (or the total amount of DNA extracted from soil) to monitor the *S. coelicolor* population in liquid cultures or soil. In a previous study using liquid cultures, the induction of several chitinase genes was successfully detected using *S. coelicolor* A3(2) mycelia grown in LB medium (Saito *et al.*, 2000). However, the results of the present study indicated that spores were more suitable than mycelia for the inoculation of *S. coelicolor* A3(2) into soil (Fig. 1a and b). The inoculation of mycelia grown in a rich medium into soil might induce orderly death or lysis of cells (Wang & Crawford, 1989).

In a previous study, the expression of several chitinase genes, including *chiA*, *B*, *C*, *D* and *F*, was demonstrated by Northern blot hybridization (Saito *et al.*, 2000). In this study, the induction of some putative chitinase genes such as *chiH*, *I*, *J* and *M*, which had not been detected previously on Northern hybridization, was also detected by real-time PCR (Fig. 2a). This may have been due to the high sensitivity of the real-time RT-PCR analysis.

The most striking difference between the chitinase expression patterns in autoclaved soil and those in liquid cultures was the high levels of chiC and chiH transcripts (especially chiH) in soil. This high induction of *chiC* and *H* was also found in nonautoclaved soil cultures in the presence of colloidal chitin (Fig. 2b). These findings indicated that the higher expression of *chiC* and *H* compared with the other chitinase genes in soil was not affected by autoclaving. A lower level of chitinase gene expression normalized as to total RNA was observed in soil that had not been autoclaved (Fig. 2b). This might be a result of the dilution of transcripts of *S. coelicolor* A3(2) with RNA derived from indigenous microorganisms. Even in the absence of *S. coelicolor* A3(2), *chiC* and *H* copies, which seemed to be derived from their orthologues in indigenous streptomycetes, could be detected in nonautoclaved soil (Fig. 2b). This fact suggests these *chiC and H* orthologues are generally induced in streptomycetes in the soil environment and play an important role in the biodegradation of chitin in the soil ecosystem.

Higher induction of other chitinases (*chiD*, *F*, *J* and *M*) was also observed in autoclaved soil (Fig. 4). These data suggest that soil contains some factors that enhance the induction of chitinase genes in the presence of colloidal chitin. For example, Suzuki *et al.* (2006) found that a chitinase inhibitor, allosamidin, which has a unique

pseudotrisaccharide structure that mimics chitin, strongly promoted the transcription and production of *chi65 Streptomyces* sp. AJ9463 in the presence of *N,N′*-diacetylchitobiose. However, allosamidin alone did not induce the production of chitinases. It has been shown that allosamidin can also influence the gene expression of chitinases in various species of streptomycetes including *chiC* in *S. coelicolor* (Homerova *et al.*, 2002; Suzuki *et al.*, 2008). An allosamidin-like factor for some chitinase genes of *S. coelicolor* might have existed in the soil. Indeed, it is expected that soil contains various chitinous polymers with different molecular weights and modifications derived from fungal cell walls and arthropod exoskeletons. Evaluation of the effects of different chitinous substrates has demonstrated that the production of *exochi*O1 chitinase of *Streptomyces olivaceoviridis* was higher in soil supplemented with crab chitin than with fungal mycelia (Vionis *et al.*, 1996). Moreover, it was shown that the combination of two chitinous substrates (colloidal chitin and fungal cell wall) enhanced chitinase production in various streptomycetes species (Gonzalez-Franco *et al.*, 2003). In addition to the above examples, other unknown factors may enhance chitinase gene expression in *Streptomyces* growing in soil. However, there is currently no information about such enhancers of chitinase gene expression in soil. The detection of such factors in the complicated and complex soil environment would certainly be interesting for future research.

In this study, the gene expression of each chitinase was precisely detected in a *dasR*-disrupted mutant, YU1, for the first time using real-time RT-PCR. Disruption of the *dasR* gene caused reductions in the induction levels of most chitinase genes in both soil and liquid cultures. Moreover, the induction level of *dasR* is strongly increased by the addition of colloidal chitin in both soil and liquid cultures (Fig. 4). In another *dasR* mutant (BAP29), the total chitinase activity was also reduced (Rigali *et al.*, 2008). Hence, we conclude that DasR is an activator for chitinase genes. In contrast to chitinase genes, DasR acts as a repressor for *pts* genes (*ptsH*, *ptsI*, *crr*, *malX2* and *nagE2*), and *nagB* (glucosamine 6-phosphase isomerase) in *N*-acetylglucosamine metabolism. Also, two activator genes, *accII-4* and *redZ*, for antibiotic production are downregulated by DasR (Rigali *et al.*, 2006, 2008). These data suggest that DasR acts as a dual regulator, which binds to the promoter regions of various genes, and acts as both an activator and a repressor for different genes. The existence of dual regulators was also found in other microorganisms, such as DtxR (iron-dependent transcriptional regulator) and MtrA (cell wall metabolism or osmoregulation) in *Corynebacterium glutamicum* (Brune *et al.*, 2006; Brocker & Bott, 2006), and Rap1p in *Saccharomyces cerevisiae* (Bendjennat & Weil, 2008).

The elevated gene expression of some chitinases (especially in the cases of *chiA*, *C* and *H*) observed in soil may reflect the higher expression of *dasR* in the soil cultures than that in the liquid cultures (Fig. 4). However, the dependence of the induction on *dasR* differed for each chitinase. Specifically, DasR is essential for the induction of *chiH* gene expression (Fig. 4). The disruption of *dasR* affected other chitinases, but not to the same degree as for *chiH*, indicating the existence of other regulator(s) for the regulation of other chitinase genes. In fact, several regulators involved in the regulation of chitinase genes have been identified in streptomycetes such as the two-component system regulator ChiS/ChiR of *chiC* in *S. coelicolor* A3(2) (Homerova *et al.*, 2002), the Cpb1 DNA-binding protein for *chiA* of *S. lividans* (Fujii *et al.*, 2005) and Reg1 of *S. lividans*, which is identical to the MalR regulator of *S. coelicolor* A3(2) (Nguyen *et al.*, 1997; Nguyen, 1999). Based on these findings, we speculated the possibility of multilevel or multiple regulation systems for the chitinase genes of *S. coelicolor* A3(2) and for the dual transcriptional regulation of the genes by DasR.

A member of subfamily C of the family 18 chitinase genes, *chiH*, was cloned and expressed in a *Streptomyces* expression system. The purified 49-kDa protein showed chitinase activity toward colloidal chitin (Fig. 5) and 4-MU-$(GlcNAc)_3$ (Table 3), indicating that *chiH* encodes an active chitinase enzyme. It was also revealed that ChiH is expressed in the presence of chitin and repressed by glucose (Fig. 5). ChiH showed approximately 80% of its maximum activity at pH 2–6.5 (Fig. 6a). The ChiH enzyme retained its activity after incubation in a wide range of acidic pHs 2–6 (Fig. 6b). Such strong stability, especially under acidic conditions, has not been found for other chitinases purified from streptomycetes (Miyashita *et al.*, 1991). Previous studies showed that streptomycetes could degrade chitinous compounds in acidic soil (Williams & Robinson, 1981). Therefore, ChiH exhibiting strong acidic pH stability might facilitate the degradation of chitinous compounds by *S. coelicolor* A3(2) under acidic soil conditions. Although the measured pH value of the soil was not so acidic (pH6.6), it can be reasonably expected that the pH distribution in soil is rather wide because of the nonhomogenous structure and content of soil. The higher induction of *chiH* with a wide range of pH stability will be advantageous for *S. coelicolor* A3(2) to degrade chitin in the soil environment.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Gene expression of *chiH* and *dasR* in the wild type and mutants of *Streptomyces coelicolor*.
Fig. S2. Molecular mass estimation of ChiH.

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