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Chitinase Inhibitor Allosamidin Is a Signal Molecule for Chitinase Production in Its Producing *Streptomyces*

II. Mechanism for Regulation of Chitinase Production by Allosamidin through a Two-component Regulatory System

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Abstract In *Streptomyces* sp. AJ9463, a producer of chitinase inhibitor allosamidin, allosamidin strongly enhances production of the chitinase mainly secreted to the culture broth in a chitin medium. To clarify the mechanism for regulation of the chitinase production by allosamidin, a disruption experiment of genes encoding proteins constructing a two-component regulatory system present at 5′-upstream region of the chitinase gene was performed. In the disruptant obtained, allosamidin could not promote the chitinase production, but *N*,*N*′-diacetylchitobiose, which also enhances production of the same chitinase more weakly than allosamidin, promoted the chitinase production similarly to the case observed in the wild-type strain. Furthermore, by the experiment in an inorganic salt solution, it was shown that allosamidin could not induce the chitinase production without addition of *N*,*N*′-diacetylchitobiose. These results show that allosamidin can activate transcription of the chitinase gene through the two-component regulatory system in the presence of *N*,*N*′-diacetylchitobiose.

Keywords allosamidin, chitinase, *Streptomyces*, *N*,*N*′-diacetylchitobiose, two-component regulatory system

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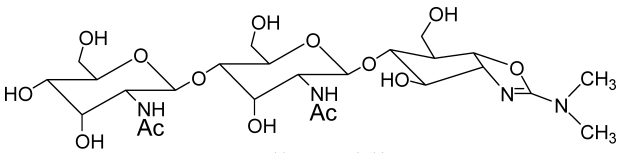
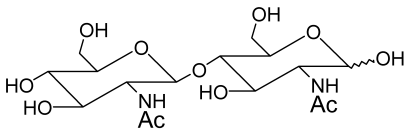
Introduction

We isolated allosamidin as the first chitinase inhibitor from the mycelia of *Streptomyces* two decades ago [1]. It has a unique pseudotrisaccharide structure (Fig. 1) and inhibits all family 18 chitinases by mimicking an intermediate in the transition state of the enzyme reaction [2, 3]. We have been investigating a role of allosamidin in its producing *Streptomyces*, and recently showed that allosamidin can dramatically promote chitinase production and growth of its producer, *Streptomyces* sp. AJ9463, in a chitin medium [4]. The chitinase whose production was enhanced by allosamidin was identified and it was shown that two genes encoding proteins constructing a two-component regulatory system are present at 5′-upstream region of the chitinase



allosamidin



N, N'-diacetylchitobiose

Fig. 1 Structures of allosamidin and *N*,*N*′-diacetylchitobiose.

gene, named *chi65*. Furthermore, we found a phenomenon that allosamidin was released from the mycelia of its producer by responding to chitin. These facts may strongly suggest that allosamidin acts as a signal molecule for chitinase production through the two-component regulatory system in its producing microbe, which is very important for the bacterial growth in chitin-rich environment such as in soil.

With respect to the regulation mechanism for gene expression of chitinases in *Streptomyces*, a direct repeat sequence present in the promoter region of the gene is known to be important for its regulation [5]. In the regulation, a repressor-type protein which can bind the sequence is assumed to be present, but the repressor is still unknown [6]. Two chitinase genes, *chi40* of *Streptomyces thermoviolaceus* [7] and *chiC* of *S. coelicolor* [8], are known to have genes encoding a two-component regulatory system in their 5′-upstream region similarly to the case of *chi65* in strain AJ9463. However, the regulation mechanism for chitinase production by the two-component system is not clarified in either case. On the other hand, *N*,*N*′-diacetylchitobiose (Fig. 1), a main product of enzymatic action of chitinase on chitin, is known as an inducer for chitinase production in *Streptomyces* [9]. The relationship between *N*,*N*′-diacetylchitobiose and the regulation at the direct repeat sequence or by the two-component regulatory system is also unknown. Therefore, it is necessary to check the effects of allosamidin and *N*,*N*′-diacetylchitobiose in parallel for investigating regulation mechanism of chitinase production by allosamidin in strain AJ9463.

In this report, we will describe the results obtained by a gene disruption experiment and an experiment in an inorganic salt solution, which showed that allosamidin can enhance the chitinase production through the two-component regulatory system in the presence of *N*,*N*′-diacetylchitobiose.

Materials and Methods

Effect of *N*,*N*′-Diacetylchitobiose on Chitinase Production

Detailed methods for culture of *Streptomyces* sp. AJ9463, detection of chitinase activity and activity staining were described in the preceding paper [4]. *N*,*N*′-Diacetylchitobiose was dissolved in 0.1 M acetic acid, and each solution was passed through a 0.25 μm sterile filter before use.

Gene Disruption

The region corresponding to the sequence upstream from the start codon (GTG) of *chi65S* encoding a sensor histidine kinase (AB239767) [4] was amplified by PCR with primers [chi65S-Uf, 5′-ttttgaattcCGCTGCCGGGGGTGCTGAT-3′ (underline: *Eco*RI site); chi65S-Ur, 5′-ttttggatccCTCCCATCCAACACGGCTGC-3′ (underline: *Bam*HI site)] and genomic DNA. The obtained 1.7 kb DNA fragment was digested with *Eco*RI and *Bam*HI, and ligated between the same sites of pUC18 to obtain pC65SR1. Similarly, the region corresponding to the sequence downstream from the stop codon (TGA) of *chi65R* encoding a response regulator (AB239767) [4] was amplified by PCR with primers [chi65R-DUf, 5′-ttttggatccTGACGTTTAGCGGCGAACTTT-3′ (underline: *Bam*HI site); chi65R-Dr, 5′-ttttaagcttAAGCCGATGCCGAGCAGCAG-3′ (underline: *Hin*dIII site)] and genomic DNA. The obtained 1.7 kb DNA fragment was digested with *Bam*HI and *Hin*dIII, and ligated between the same sites of pC65SR1 to obtain pC65SR2, which contained both *Eco*RI-*Bam*HI and *Bam*I-*Hin*dIII inserts. In the *Bam*HI site between the two inserts in pC65SR2, a 1.9 kb *Bam*HI fragment containing the neomycin resistance gene *aphII* from pAPH3k [10] was ligated to obtain pΔchi65SR. This vector was linearized with *Dra*I and denatured with 0.1 M NaOH, which was introduced by protoplast transformation into *Streptomyces* sp. AJ9463 [11]. Correct replacement of the disrupted *chi65SR* sequence was checked by Southern hybridization with a probe (Fig. 4A) from *aphII* against the *Apa*I-digested chromosomal DNA to detect a 5.1 kb *Apa*I fragment in the *Δchi65SR* strain (Fig. 4B). Disruption of chi65SR was confirmed by RT-PCR (Fig. 4C). For complementation of the *chi65SR* disruption, a 2.0 kb DNA fragment containing the whole *chi65SR* (DNA fragment for complementation analysis in Fig. 4A) was amplified by PCR with primers [chi65SR$^+$-f, 5′-ttttgaattcGGCACTTCCCCTTTCCGTCG-3′ (underline: *Eco*RI site); chi65SR$^+$-r, 5′-ttttaagcttAAGTTCGCCGCTAAACGTCA-3′ (underline: *Hin*dIII site)] and genomic DNA. The obtained 2.0 kb DNA fragment was digested with *Eco*RI and *Hin*dIII, and ligated between the same sites of pUWL-KS to obtain pCHI65SR$^+$. Expression of *chi65SR* in *chi65SR*$^+$ strain was confirmed by RT-PCR (Fig. 4C).

RT-PCR

Total RNA was extracted from the mycelia of strain AJ9463 with SV Total RNA Isolation System (Promega) according to the manufacturer's procedure. First-strand cDNA was reversetranscribed using the random hexamer and SuperScript TM First-Strand Synthesis System (Invitrogen) from 500 ng of the total RNA. PCR amplification was performed with pairs of primers (chi65-f, 5′-GTGACTCCTACGCCGACTA-3′, and chi65-r, 5′-GTTGTTGCCGC-

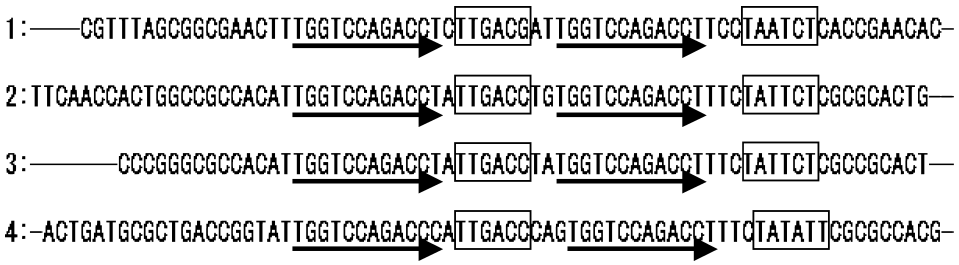


Fig. 2 Nucleotide sequences of the promoter regions of *chi65* (1), *chi63* of *S. plicatus* (2), *chiC* of *S. coelicolor* (3) and *chi40* of *S. thermoviolaceus* (4).

Solid arrows indicate direct repeat sequences. Putative regions interacting with RNA polymerase were boxed.

CGAACTT-3′, for *chi65*; chi65S-f, 5′-GTTCACGGAC-CTCATGTACGCGGC-3′, and chi65S-r, 5′-ACGGGCC-ATCCGGGTGCGCTCG-3′, for *chi65S*; chi65R-f, 5′-CC-GCGGGCTTCCTGCTGAA-3′, and chi65R-r, 5′-GCGAG-TACGGCTGCCTGGA-3′, for *chi65R*; chi65SR-f, 5′-CG-GTGGAGGTCAGCAGTGT-3′, and chi65SR-r, 5′-AGTA-CGTCCGCGAGTTGTCC-3′ for *chi65SR*; hrdB-f, 5′-AGGTCGAGCTCGCCAAGCGGATC-3′, and hrdB-r, 5′-GAGCTTGTTGATGACCTCGACCAT-3′ for *hrdB*) and 1/20～1/300 aliquot of these first-strand cDNA as a template.

Chitinase Production in an Inorganic Salt Solution

Spores of strain AJ9463 were inoculated into a Bennet medium (100 ml) in a 500-ml Erlenmeyer flask, and the flask was incubated at 30°C and 150 rpm on a rotary shaker for 23 hours. Mycelia obtained from this culture, which did not contain allosamidin, were suspended in an inorganic salt solution (400 ml) consisting of 0.05% KCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$ (pH 7.2) and shaken at 30°C and 120 rpm on a rotary shaker for 30 minutes. After recollecting the washed mycelia by centrifugation (3,000 *g*×5 minutes), the mycelia were resuspended in the inorganic salt solution (400 ml). Allosamidin and/or *N*,*N*′-diacetylchitobiose was added to this suspension (50 ml) in a 500-ml Erlenmeyer flask, which was incubated at 30°C and 120 rpm on a rotary shaker for 2 hours.

Results

Presence of a Direct Repeat Sequence in the Promoter of *chi65*

Allosamidin promotes production of chitinases derived from *chi65* in a chitin medium in strain AJ9463. The promoter region of *chi65* contained a direct repeat sequence. Homologous sequences are widely observed in promoter regions of known chitinase genes of *Streptomyces* (Fig. 2), which include *chi40* and *chiC* with genes of a two-component regulatory system in their 5′-upstream region [12].

Effect of *N*,*N*′-Diacetylchitobiose on Chitinase Production

Figure 3A shows dose-response of *N*,*N*′-diacetylchitobiose's effect on the chitinase production by strain AJ9463 when the strain was cultured in a chitin medium containing chitin as the sole carbon source. Allosamidin's effect tested at the same time is also shown for comparison in Fig. 3A. *N*,*N*′-Diacetylchitobiose could increase the chitinase activity of the culture filtrate, but its activity was weak and different from that of allosamidin. Higher concentration of *N*,*N*′-diacetylchitobiose than that in the case of allosamidin was necessary to increase the chitinase activity in the culture filtrate, and the enhancement level of chitinase activity caused by *N*,*N*′-diacetylchitobiose was much lower than that by allosamidin. The patterns of chitinases in each culture filtrate detected by activity staining after SDS-PAGE are shown in Fig. 3B. *N*,*N*′-Diacetylchitobiose activated the production of the same chitinases at 105 kDa and 46 kDa as observed in the case of allosamidin (Fig. 3B). The two chitinases had been shown to be originated from the same gene, *chi65* [4].

Gene Disruption Experiment

Two genes, *chi65S* and *chi65R*, encoding a sensor histidine kinase and a response regulator, respectively, are present at the 5′-upstream of *chi65* (Fig. 4A). To clarify the function of CHI65S and CHI65R for *chi65* expression, a gene disruption experiment was carried out to obtain the *Δchi65SR* strain in which both *chi65S* and *chi65R* were inactivated (Fig. 4). For a complementation experiment, the *chi65SR*$^+$ strain with a plasmid containing *chi65S* and *chi65R* was also prepared by introducing the plasmid into the *Δchi65SR* strain. The patterns of chitinase production in

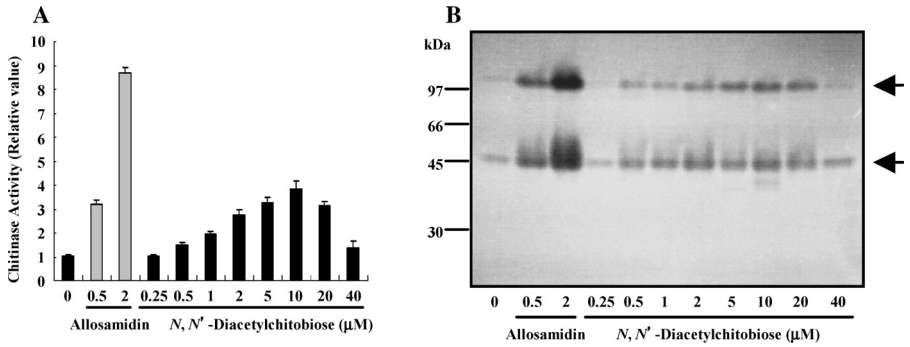


Fig. 3 Effects of *N*, *N*′-diacetylchitobiose and allosamidin on chitinase production of *Streptomyces* sp. AJ9463.

Chitinase activity of the culture filtrate after 24 hours of cultivation in a chitin medium with *N*, *N*′-diacetylchitobiose or allosamidin was measured with 4-methylumbelliferyl-*N*, *N*′, *N*′-triacetyl chitotrioside [4MU-$(GlcNAc)_3$] as a substrate (A) or by activity staining on a chitin-containing gel (B). In (A), the values of relative chitinase activity were calibrated based on the activity of a control without *N*, *N*′-diacetylchitobiose or allosamidin and means±S.E. were shown (N=3). Arrows in (B) indicate the bands of 105 kDa and 46 kDa proteins.

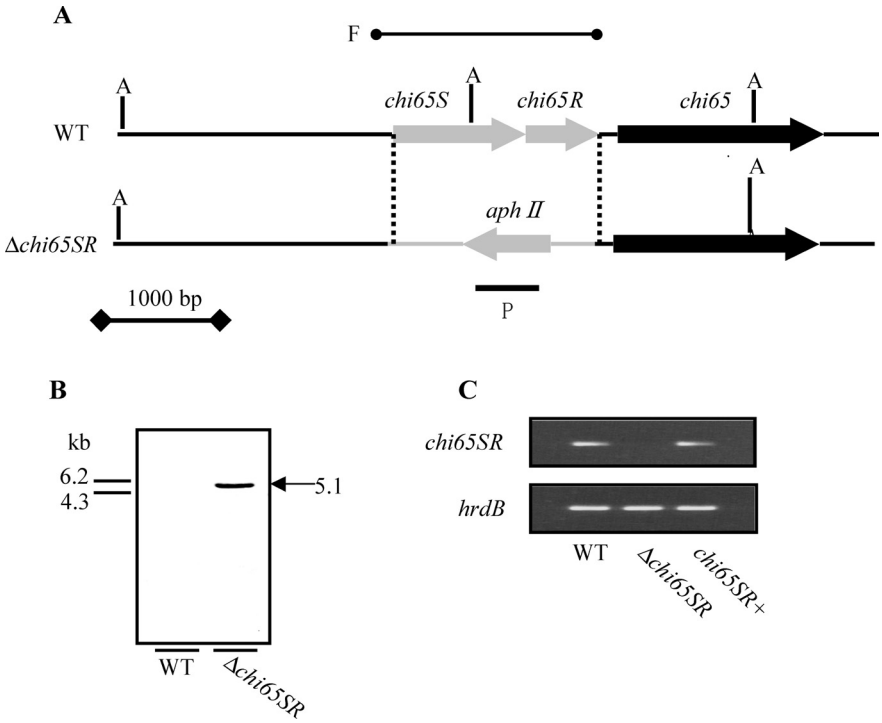


Fig. 4 Gene disruption experiment.

(A) Construction of *Δchi65SR* strain. The stop codon (TGA) of *chi65S* and the start codon (ATG) of *chi65R* were overlapped (ATGA) at the junction of the two genes. A, *Apa*I site. P, probe. F, DNA fragment on pCHI65SR$^+$ used for complementation analysis. (B) Southern hybridization to check correct disruption with the probe in (A) against the *Apa*I-digested chromosomal DNA. (C) Expression of *chi65SR* detected by RT-PCR in wild type, *Δchi65SR* and *chi65SR*$^+$ strains.

the culture filtrates of these two and wild-type strains were examined under the conditions with or without allosamidin or *N*, *N*′-diacetylchitobiose (Fig. 5). In the *Δchi65SR* strain, allosamidin did not activate production of 46 kDa and 105 kDa chitinases derived from *chi65*, but the response was recovered in the *chi65SR*$^+$ strain (Fig. 5A). On the other hand, *N*, *N*′-diacetylchitobiose activated production of the chitinases in the *Δchi65SR* strain as observed in the wild-type strain (Fig. 5B). Next, the mRNA levels of *chi65* in the *Δchi65SR*, *chi65SR*$^+$ and wild-type strains were examined (Fig. 6). In the wild type strain, the mRNA level of each *chi65S* and *chi65R* was not affected by allosamidin

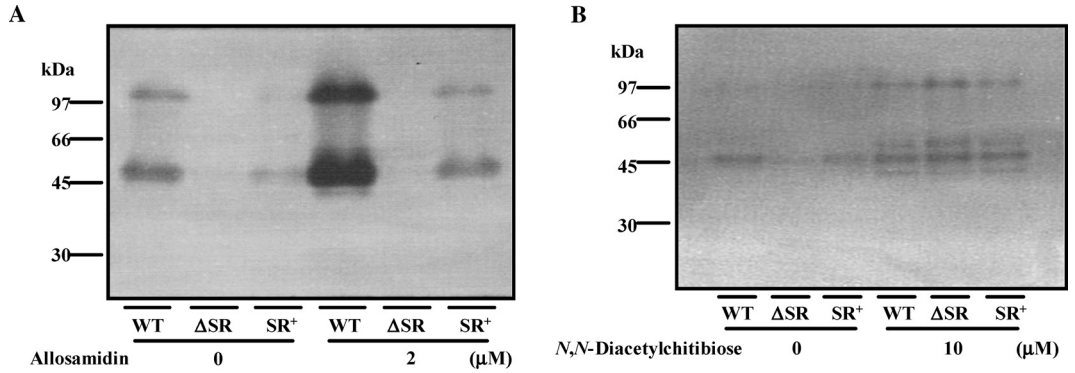


Fig. 5 Effects of allosamidin and *N*, *N*′-diacetylchitobiose on chitinase production of wild-type (WT), *Δchi65SR* (ΔSR) and *chi65SR*$^+$ (SR$^+$) strains.

Three strains were cultured in a chitin medium with or without allosamidin (A) or *N*, *N*′-diacetylchitobiose (B). After 24 hours of cultivation, chitinase activity in each culture filtrate was detected on a chitin-containing gel by activity staining.

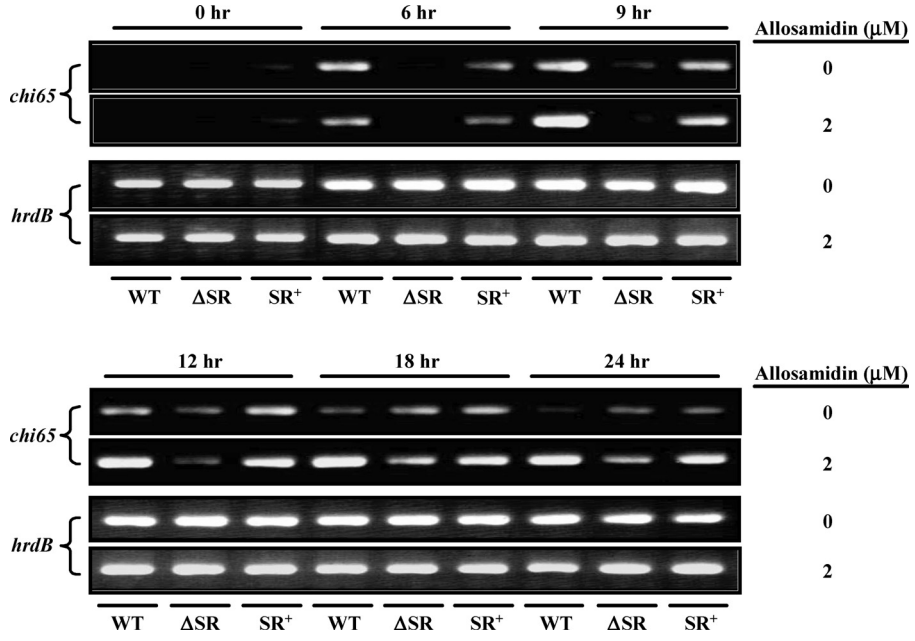


Fig. 6 Effect of allosamidin on mRNA levels of *chi65*.

Wild-type (WT), *Δchi65SR* (ΔSR) and *chi65SR*$^+$ (SR$^+$) strains were cultured in a chitin medium with or without allosamidin. The mRNA levels of *chi65* and *hrdB*, a housekeeping gene, in mycelia obtained from each culture broth were measured by RT-PCR at each cultivation time (0, 6, 9, 12, 18 or 24 hours).

(Fig. 7), but that of *chi65* in the culture with allosamidin was maintained at a higher level than that of the control without allosamidin for a long time (Fig. 6). The transcription of *chi65* was not activated by allosamidin in the *Δchi65SR* strain, and recovery was observed in the *chi65SR*$^+$ strain (Fig. 6). On the other hand, *N*, *N*′-diacetylchitobiose activated the transcription of *chi65* in the *Δchi65SR* strain as well as the wild-type strain (Fig. 8). These results indicated that allosamidin activated *chi65* expression through the two-component system of CHI65S and CHI65R, but *N*, *N*′-diacetylchitobiose did so without using this system.

Induction of Chitinase Production in an Inorganic Salt Solution

To clarify each function of allosamidin and *N*, *N*′-diacetylchitobiose in the event of the *chi65* expression, allosamidin's effect was tested using an *N*, *N*′-

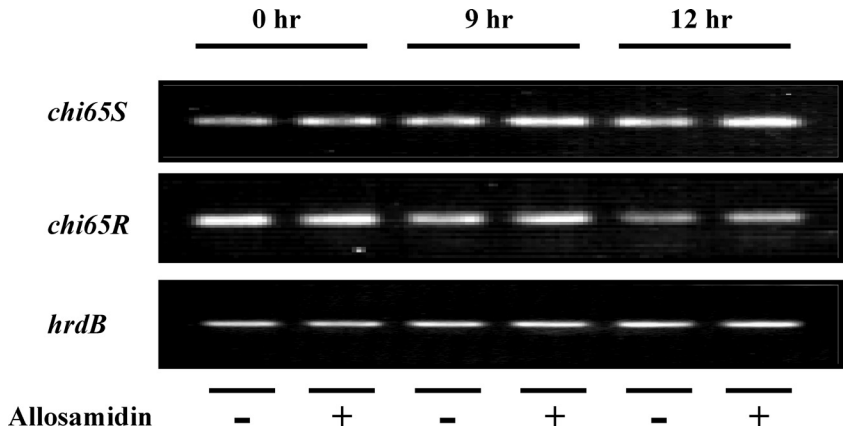


Fig. 7 Effect of allosamidin on mRNA levels of *chi65R* and *chi65S*.

Strain AJ9463 was cultured in a chitin medium with or without allosamidin (2 μM). The mRNA levels of *chi65S*, *chi65R* and *hrdB*, a housekeeping gene, in mycelia were measured by RT-PCR at each cultivation time (0, 9 or 12 hours).

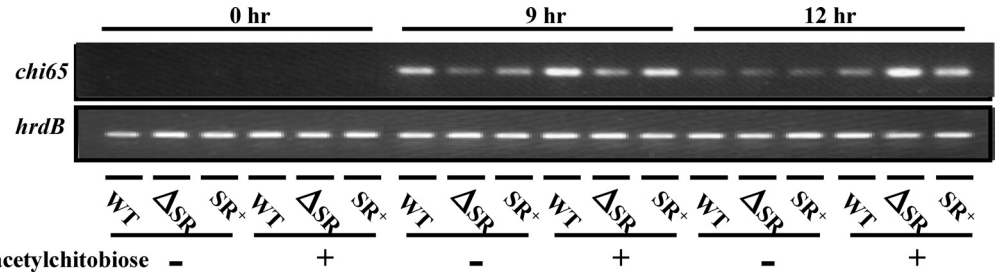


Fig. 8 Effect of *N,N′*-diacetylchitobiose on mRNA levels of *chi65*.

Wild-type (WT), *Δchi65SR* (ΔSR) and *chi65SR*$^+$ (SR$^+$) strains were cultured in a chitin medium with or without *N, N′*-diacetylchitobiose (10 μM). The mRNA levels of *chi65* and *hrdB*, a housekeeping gene, in mycelia obtained from each culture broth were measured by RT-PCR at each cultivation time (0, 9 or 12 hours).

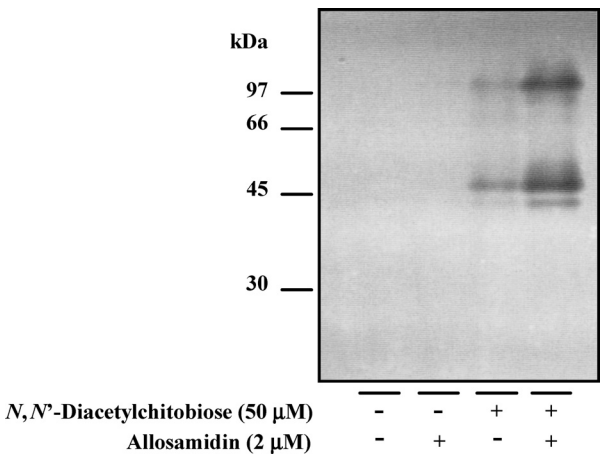


Fig. 9 Effects of allosamidin and *N,N′*-diacetylchitobiose on chitinase production in an inorganic salt solution.

Mycelia of the wild-type strain cultured in a Bennet medium were incubated in an inorganic salt solution with allosamidin (2 μM) and/or *N,N′*-diacetylchitobiose (50 μM) for 2 hours. Chitinase activity of each supernatant was detected on a chitin-containing gel by activity staining.

diacetylchitobiose-free inorganic salt solution because N,N'-diacetylchitobiose was present constantly in the culture broth by the action of chitinases when strain AJ9463 was growing in a chitin medium. Mycelia of strain AJ9463 cultivated in a chitin-free medium, in which allosamidin was not present and both *chi65R* and *chi65S* were expressed, were gathered and resuspended in an inorganic salt solution. When the cell suspension was incubated with N,N'-diacetylchitobiose, production of 46 and 105 kDa chitinases was induced (Fig. 9). Allosamidin by itself could not solely induce production of the chitinases, but it strongly promoted the chitinase production induced by N,N'-diacetylchitobiose (Fig. 9). This indicated that allosamidin could activate *chi65* transcription under the presence of N,N'-diacetylchitobiose.

Discussion

In this study, we investigated the regulatory mechanism for

chitinase-production promoting activity by allosamidin based on the information of DNA sequence in the 5′-upstream region of *chi65* encoding chitinases whose production is enhanced by allosamidin. Presence of the direct repeat sequence at the promoter region of *chi65* and two genes encoding a two-component regulatory system suggested that regulation of *chi65* expression is not simple. In *Streptomyces plicatus*, it was shown that the direct repeat sequence region at the promoter of *chi63* was essential for regulation of the gene expression by glucose repression [5]. Therefore a repressor-type protein is assumed to bind the region of the direct repeat sequence for the regulation, but its detailed mechanism is still unknown. *N*,*N*′-Diacetylchitobiose, which is known as an inducer for chitinase production in *Streptomyces* [9], weakly enhanced production of chitinases derived from *chi65* in strain AJ9463 and its enhancement activity was not changed in the *Δchi65SR* strain lacking two genes for the two-component regulatory system. Furthermore, it was shown that *N*,*N*′-diacetylchitobiose acts as an inducer for production of chitinases originated from *chi65* in an inorganic salt solution. From these facts, it may be possible to speculate that *N*,*N*′-diacetylchitobiose induces the *chi65* transcription by a regulation mechanism through the direct repeat sequence at the promoter region of *chi65*.

In the *Δchi65SR* strain, allosamidin could not activate the *chi65* transcription, indicating that allosamidin does not have such action at the direct repeat sequence as *N*,*N*′-diacetylchitobiose has and the two-component regulatory system is necessary for allosamidin's action on chitinase production. Allosamidin did not act as an inducer for the chitinase production in an inorganic salt solution, but it acted as an activator in the presence of *N*,*N*′-diacetylchitobiose. From the results obtained, we could imagine the following mechanism for the *chi65* expression (Fig. 10). First, *N*,*N*′-diacetylchitobiose induces the *chi65* expression by an unknown mechanism to derepress the regulation concerning the direct repeat sequence moiety, and then allosamidin dramatically activates it by directly attaching the sensor moiety of the two-component regulatory system or through an unknown mechanism leading to activation of the two-component system. Studies to investigate generality of the allosamidin's action in *Streptomyces* and interaction of allosamidin and *N*,*N*′-diacetylchitobiose to their binding proteins in the chitinase production system are now in progress.

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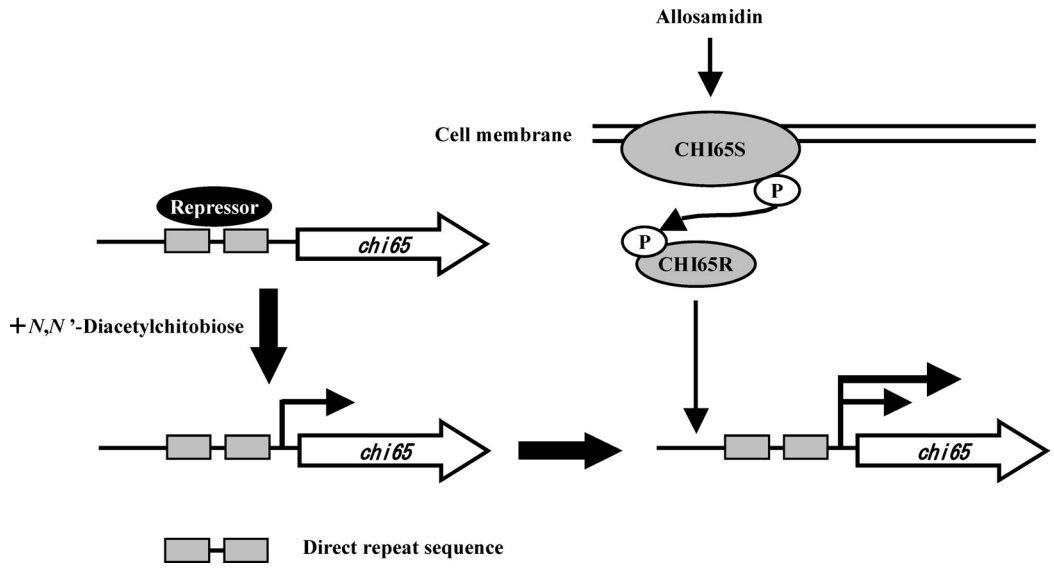

Fig. 10 Putative mechanism for *chi65* expression.

N,*N*′-Diacetylchitobiose may derepress the regulation concerning the direct repeat sequence and a putative repressor protein to induce the *chi65* expression. After the derepression, allosamidin can dramatically activate the *chi65* expression through the two-component regulatory system of CHI65S and CHI65R.

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