CHITINASE INHIBITOR ALLOSAMIDIN IS A SIGNAL MOLECULE FOR CHITINASE PRODUCTION IN ITS PRODUCING STREPTOMYCES

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Abstract

Allosamidin, a typical secondary metabolite of *Streptomyces*, has been known as a chitinase inhibitor. We found that allosamidin can dramatically promote chitinase production and growth of its producing microorganism in a chitin medium. Allosamidin was released from the microbial mycelia by responding to chitin and strongly activated transcription of a gene encoding the main chitinase secreted to the culture broth through a two-component regulatory system in the presence of *N*, *N'*-diacetylchitobiose. This shows that allosamidin acts as a key signal molecule for chitinase production in its producing strains, which may be useful for their growth in chitin-rich environment.

Introduction

Microorganisms produce a large number of secondary metabolites with a variety of structures and biological activities. Many of them are now absolutely necessary for our life as useful compounds such as medicines or pesticides. The variety of secondary metabolisms present in microorganisms suggests that they have been developing them during evolution and each secondary metabolite might have a significant role in its producer under some circumstances. The role of antibiotic production is presumable but has not been proved. It is entirely unknown why microorganisms produce many other compounds without antibiotic activity such as enzyme inhibitors.

Streptomyces, a soil bacterium, is one of the most important microorganisms as a producer of useful secondary metabolites. Allosamidin is a typical secondary metabolite of *Streptomyce*s without antibiotic activity [1]. It has a unique pseudotrisaccharide structure (Fig. 1) mimic to chitin [2] and inhibits all family 18 chitinases, which hydrolyze chitin and are widely present in nature [3]. Allosamidin has been used to investigate the physiological role of chitinases involved not only in chitin-containing organisms, but also in non-chitin-containing ones. In the former case, it inhibits cell separation in yeast [4], transmission of the malaria parasite [5] and insect molting [1]. In the latter case, inhibition of acidic mammalian chitinase by allosamidin causes a decrease in asthmatic Th2 inflammation [6].

With respect to allosamidin and its producer, we found a phenomenon that chitinase production in a chitin medium by the producer was activated by addition of allosamidin exogenously [7]. This seems to indicate that allosamidin has a physiological role in its producing *Streptomyces* because chitinase production is very important for the bacterial growth in soil where chitin originated mainly from insect cuticle and fungal cell walls is a major nutrient source. Here, we describe our recent data to prove this hypothesis at the molecular level [8, 9].

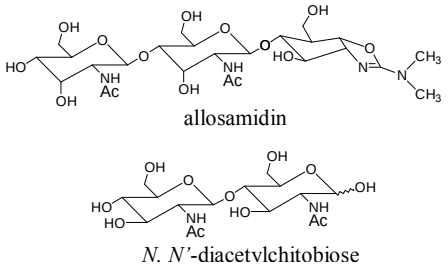

Figure 1 : Structures of allosamidin and *N, N'*-diacetylchitobiose.

Materiel and Methods

Culture

Streptomyces sp. AJ9463, an allosamidin producer [10], was maintained on a Bennet agar medium consisting of glucose (1%), peptone (0.2%), meat extract (0.1%), and yeast extract (0.1%) (pH 7.2) at 30 °C and subcultured at about monthly intervals. Spores of the strain 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 46 h. This preculture was used for all main cultures in this study. In the experiments with a chitin medium which consists of colloidal chitin (0.2%), KCl (0.05%), K_2HPO_4 (0.1%), $MgSO_4$ (0.05%) and $FeSO_4$ (0.001%) (pH 7.2), the preculture (1.4 mL) was transferred into the chitin medium (70 mL) in a 500-mL Erlenmeyer flask, and the flask was incubated at 30 °C and 120 rpm on a rotary shaker. Allosamidin and *N*, *N'*-diacetylchitobiose were dissolved in 0.1 M acetic acid, and each solution after being passed through a 0.25 μm sterile filter before use was added to the chitin medium or an inorganic salts solution at the beginning of culture.

Activity staining

The culture supernatant (10 mL in the experiments with a chitin medium or 50 mL in those with an inorganic salts solution) in each experiment was concentrated by ultrafiltration (10,000 cut membrane, AMICON) to 4 mL. Acetone (12 mL) was added to the concentrated solution to afford a precipitate. The precipitate was collected by centrifugation (7,000 *g*, 30 min) and dissolved in distilled water (30 μL), followed by mixing with the electrophoresis sample buffer (30 μL). After being left overnight at room temperature, the mixture was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. After SDS-PAGE, the gel was washed with 0.1 M AcONa – AcOH buffer (pH 5.0) for 5 min and layered over the gel containing glycolchitin (0.01%) and polyacrylamide gel (7.5%) with or without allosamidin to transfer the proteins in the upper gel into the lower gel. The gels were left in 0.15 M AcONa – AcOH buffer (pH 5.0) with or without allosamidin at 37 °C for 180 min to allow an enzyme reaction by changing the buffer solution at 60 min intervals. The lower gel was stained by 0.01% fluorescent brightener 28 (Calcoflour, Sigma-Aldrich) in 0.1 M Tris – HCl (pH 8.8) buffer for 5 min at room temperature and washed overnight in water. The chitinase lytic bands on the gel were detected by a UV transilluminator.

Detection of growth-promoting activity of allosamidin

Since *Streptomyces* grows in a mycelial form like filamentous fungi, a value of mycelial weight or volume is usually used as an indicator for its growth rate. But, in a medium with a solid constituent

like chitin, such method can not be applied to compare growth among different cultures. Therefore, mycelia cultured in the chitin medium with or without allosamidin were directly observed under microscope in this study. Chitin contained in the culture was stained by 0.0005% fluorescent brightener 28 (Calcoflour, Sigma-Aldrich) and observed under a fluorescence microscope. The amount of ATP in each culture was measured by a luciferin-luciferase-based bioluminescence assay with a Lucifer LU plus kit (Kikkoman Co.) according to its protocol. Luminescence was measured by a luminometer (Luminescencer-JNR AB-2100, ATTO).

RT-PCR

Total RNA was extracted from the mycelia of strain AJ9463 with SV Total RNA Isolation System (Promega, Inc.) 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'-GTTGTTGCCGCCGAACTT -3', for *chi65*; chi65S-f, 5'-GTTCACGGACCTCATGTACGCGGC-3', and chi65S-r, 5'-ACGGGCCATCCGGGTGCGCTCG-3', for *chi65S*; chi65R-f, 5'-CCGCGGGCTTCCTGCTGAA-3', and chi65R-r, 5'-GCGAGTACGGCTGCCTGGA-3', for *chi65R*; chi65SR-f, 5'-CGGTGGAGGTCAGCAGTGT-3', and chi65SR-r, 5'-AGTACGTCCGCGAGTTGTCC-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 salts 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 h. Mycelia obtained from this culture, which did not contain allosamidin, were suspended in an inorganic salts solution (400 mL) consisting of 0.05% KCl, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$, 0.2% $(NH_4)_2SO_4$ (pH 7.2) and shaken at 30 °C and 120 rpm on a rotary shaker for 30 min. After recollecting the washed mycelia by centrifugation (3,000 *g*, 5 min), the mycelia were resuspended in the inorganic salts 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.

Allosamidin release from mycelia

The preculture (2.0 mL) of strain AJ9463 described above was inoculated into a Bennet medium (100 mL) in a 500-mL Erlenmeyer flask, and incubation was done at 30 °C and 150 rpm on a rotary shaker for 120 h. Mycelia was collected by centrifugation (3,000 *g*, 5 min) and washed with distilled water three times. The washed mycelia containing allosamidin (0.7 μg) was resuspended in an inorganic salts solution (50 mL) or in a chitin medium (50 mL), and the suspension was incubated at 30 °C and 150 rpm on a rotary shaker for 24 h. The amounts of allosamidin in both the mycelia and supernatant were quantified by HPLC [12].

Result and Discussion

We used an allosamidin-producing strain *Streptomyces* sp. AJ9463 in this study [10]. When the strain was cultured in a chitin medium which contains chitin as the sole carbon source, allosamidin enhanced the chitinase activity of the culture filtrate dose-dependently up to 2 μM (Fig. 2a). Clear enhancement was observed even at 60 nM. This effective concentration of allosamidin on chitinase production may be physiologically significant because the concentration of allosamidin produced in the culture broth was usually around the range of a few hundred nM to a few μM. The pattern of chitinase production in each culture filtrate was analyzed by using the activity staining after SDS-PAGE (Fig. 2b). Two major bands at 105 kDa and 46 kDa, named chitinase 105 and chitinase 46, respectively, were detected in all culture filtrates, and the staining density of both bands increased in a dose-dependent manner. The presence of the two bands in the culture filtrate of a control indicated that they were main chitinases produced by strain AJ9463 in the chitin medium. The two chitinases were inhibited by allosamidin at high concentrations of more than 10 μM, suggesting that the allosamidin's effect on chitinase production was not disturbed by its chitinase inhibitory activity at low concentrations effective on chitinase production.

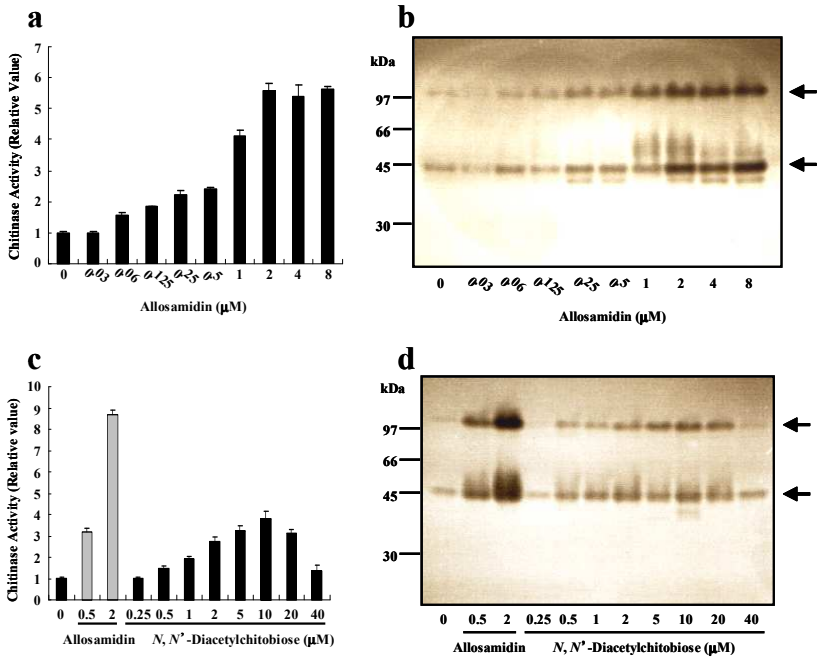

Figure 2 : Effects of allosamidin and *N*, *N'*-diacetylchitobiose on chitinase production of *Streptomyces* sp. AJ9463. Chitinase activity of the culture filtrate after 24 h of cultivation in a chitin medium with allosamidin (**a**, **b**) or *N*, *N'*-diacetylchitobiose (**c**, **d**) was measured with 4-methylumbelliferyl-*N*, *N'*,*N''*-triacetyl chitotrioside [4MU-$(GlcNAc)_3$] as a substrate (**a**, **c**) or by activity staining on a chitin-containing gel (**b**, **d**). Each culture filtrate in (**a**) and (**c**) was the same as that in (**b**) and (**d**), respectively. The same preculture was used for each series of cultures in (**a**, **b**) or (**c**, **d**). In (**a**) and (**c**), the values of relative chitinase activity, which were slightly changed according to the batch of the preculture, were calibrated based on the activity of a control without allosamidin or *N*, *N'*-diacetylchitobiose and means +/- S.E. were shown (N = 3). Arrows in (**c**) and (**d**) indicate the bands of 105 kDa and 46 kDa proteins.

In the chitin medium, chitinase production is necessary for bacterial growth and the enhancement of chitinase activity would lead to promoting the growth by accelerating chitin degradation. In fact, mycelia of strain AJ9463 observed under a microscope grew much better in the presence of

allosamidin compared to those of a control (Fig. 3a). At that time, the amount of chitin particles remaining in each culture broth, which was shown by Calcoflour staining (Fig. 3a), indicated that chitin in the culture broth with allosamidin was degraded faster than that in the control culture. ATP levels in culture broths also supported this growth-promoting effect of allosamidin (Fig. 3b).
N, *N'*-Diacetylchitobiose (Fig. 1), a main product of enzymatic action of chitinase on chitin, is known as an inducer of chitinase production in *Streptomyces lividans* [13]. It also activated chitinase production of strain AJ9463 (Fig. 2c), but its activity was weaker than that of allosamidin. Production of chitinases 105 and 46 was activated by *N*, *N'*-diacetylchitobiose as observed in the case of allosamidin (Fig. 2d).

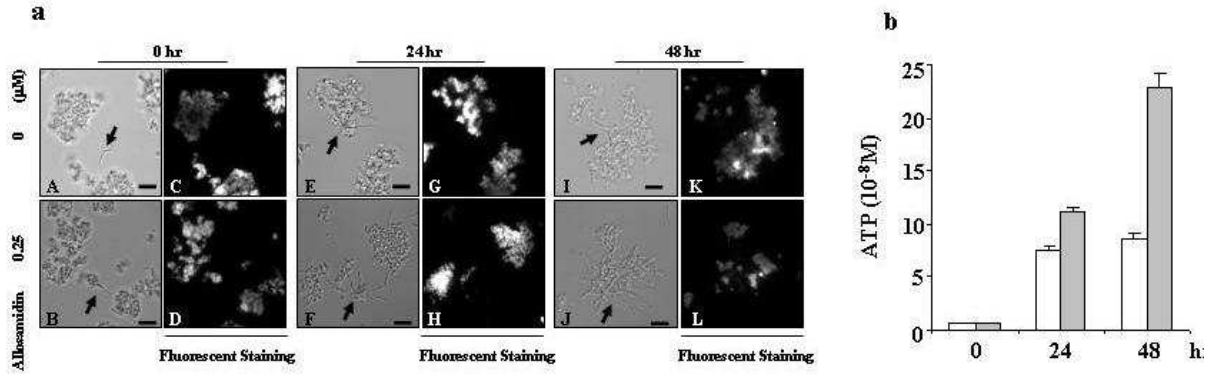

Figure 3 : Growth-promoting activity of allosamidin observed under a microscope (**a**) and estimated from ATP amounts (**b**). Strain AJ 9463 was cultured in a chitin medium with (B, D, F, H, J, L, gray bars) or without 0.25 μM of allosamidin (A, C, E, G, I, K, white bars) for 0 h (A, B, C, D), 24 h (E, F, G, H) or 48 h (I, J, K, L). Each pair of A and C, B and D, E and G, F and H, I and K, or J and L is the same preparation. Mycelia, which are indicated by arrows, and chitin particles in each culture broth were observed under a microscope (A, B, E, F, I, J) and a fluorescence microscope by Calcoflour staining for detection of chitin (C, D, G, H, K, L), respectively. The pattern shown in each photograph was observed over the corresponding culture broth. Scale bar, 10 μm. ATP amount in each culture broth at 0 h, 24 h or 48 h cultivation time was measured with a luciferin-luciferase-based bioluminescence assay kit.

We analyzed the N-terminal amino acid sequence of chitinase 46 and revealed that it was a degradation product of a protein encoded by a gene, named *chi65*(AB239767). Chitinase 105 afforded the same N-terminal amino acid sequence as that of chitinase 46, suggesting that chitinase 105 also originated from *chi65* and it has a dimer structure. The amino acid sequence deduced from the nucleotide sequence of *chi65* had high homology to known family 18 chitinases of *Streptomyces*. Chitinase 46 lacked the N-terminal region of the sequence. The promoter region of *chi65* possessed a direct repeat sequence like other *Streptomyces* chitinase genes [14]. The sequence is known to be essential for regulation of chitinase expression [15]. In the regulation, an unknown repressor-type protein which can attach the sequence is assumed to be present [16]. Analysis of the DNA fragment containing *chi65* revealed the presence of two genes, named *chi65S* and *chi65R* (AB239767), at the 5'-upstream region of *chi65*. The amino acid sequences deduced from the nucleotide sequences of the genes indicated that *chi65S* and *chi65R* encoded a sensor histidine kinase and a response regulator, respectively, constructing a typical two-component regulatory system (Fig. 4a). In the system, a signal molecule existing outside of the cells may attach the sensor moiety of CHI65S, a membrane protein with five membrane-spanning domains, and the signal may activate CHI65R, a DNA binding protein with a helix-turn-helix domain, by a phosphorylation relay system observed in usual two-component regulatory systems [17]. The activated CHI65R would regulate *chi65* expression. Many chitinase genes of *Streptomyces* have been obtained, but in only two cases, *chi40* of *Streptomyces thermoviolaceus* [18] and *chiC* of *Streptomyces coelicolor* [19], homologs of

chi65S and *chi65R* are present in the upstream region of each chitinase gene. Although *S. lividans* transformed with a DNA fragment containing *chi40* and two genes encoding the two-component system could produce CHI40 in the presence of chitin and a gene disruption experiment suggested that the two-component system may regulate the *chiC* expression in *S. coelicolor*, detailed mechanism for the regulation including a ligand of the sensor moiety has not been clarified in both cases.

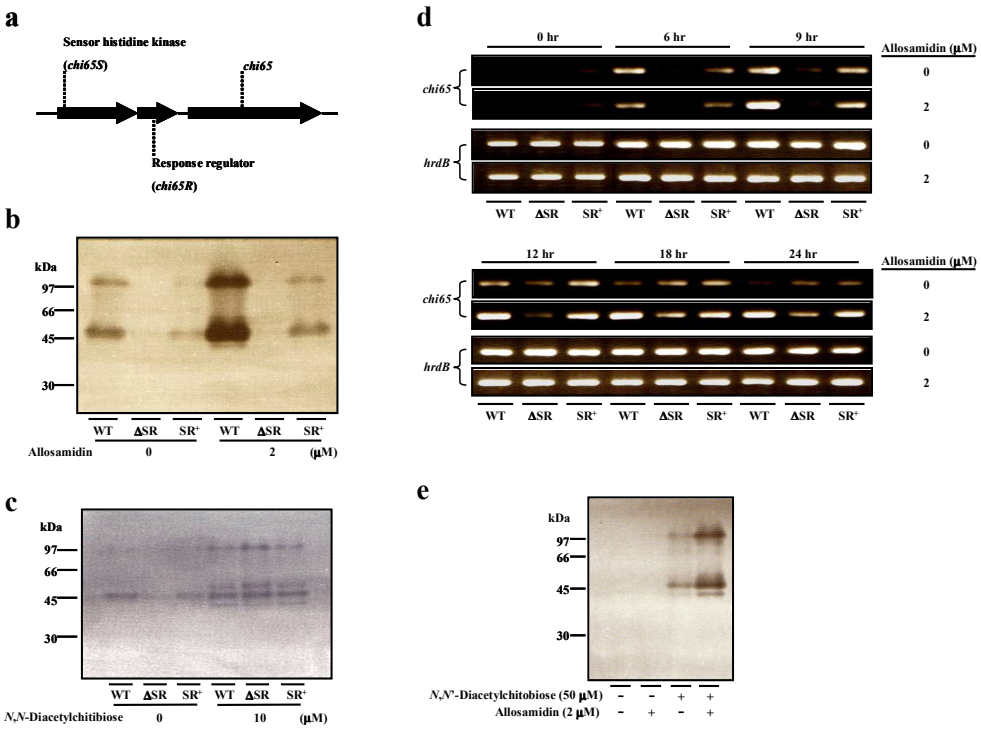

Figure 4 : Regulation of *chi65* expression by allosamidin, *N*, *N'*-diacetylchitobiose and the two-component regulatory system. (**a**) Schematic representation of *chi65*, *chi65S* and *chi65R*. The stop codon (TGA) of *chi65S* and the start codon (ATG) of *chi65R* were overlapped (ATGA) at the junction of the two genes. Wild-type (WT), *Δchi65SR* (ΔSR) and *chi65SR*$^+$ (SR$^+$) strains were cultured in a chitin medium with or without allosamidin (**b**) or *N*, *N'*-diacetylchitobiose (**c**). After 24 h of cultivation, chitinase activity in each culture filtrate was detected on a chitin-containing gel by activity staining. (**d**) 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 h, 6 h, 9 h, 12 h, 18 h or 24 h). (**e**) Mycelia of the wild-type strain cultured in a Bennet medium were incubated in an inorganic salts solution with allosamidin (2 μM) and/or *N*, *N'*-diacetylchitobiose (50 μM) for 2 h. Chitinase activity of each supernatant was detected on a chitin-containing gel by activity staining.

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 [8, 11]. 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. In the *Δchi65SR* strain, allosamidin did not activate production of chitinases 46 and 105, but the response was recovered in the *chi65SR*$^+$ strain (Fig. 4b). On the other hand, *N*, *N'*-diacetylchitobiose activated production of the two chitinases in the *Δchi65SR* strain as observed in the wild-type strain (Fig. 4c). In the wild type strain, the mRNA level of each *chi65S* and *chi65R* was not affected by allosamidin, 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. 4d). The transcription of *chi65* was not activated by allosamidin in the *Δchi65SR* strain, and recovery was observed in the *chi65SR*$^+$ strain (Fig. 4d). On the other hand, *N*, *N'*-diacetylchitobiose activated the transcription of *chi65* in the *Δchi65SR* strain as well as the wild-type strain. These results indicated that allosamidin activated *chi65* expression through the two-component system, but *N*, *N'*-diacetylchitobiose did so without using this system. It may be possible to speculate that *N*, *N'*-diacetylchitobiose induces *chi65* expression by a regulation mechanism through the above-mentioned direct repeat sequence. Since allosamidin can not activate the *chi65* transcription in the *Δchi65SR* strain, it does not have such action as *N*, *N'*-diacetylchitobiose has.

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'*-diacetylchitobiose-free inorganic salts 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, were gathered and resuspended in an inorganic salts solution. When the cell suspension was incubated with *N*, *N'*-diacetylchitobiose, production of chitinases 46 and 105 was induced (Fig. 4e). Allosamidin could not solely induce production of the chitinases, but it strongly promoted the chitinase production induced by *N*, *N'*-diacetylchitobiose (Fig. 4e). This indicated that allosamidin could activate *chi65* transcription in the presence of *N*, *N'*-diacetylchitobiose. From the results obtained, we could image the following mechanism for the *chi65* expression: first, *N*, *N'*-diacetylchitobiose induces the *chi65* expression by derepressing the regulation concerning the direct repeat sequence moiety, and then allosamidin activates it through the two-component regulatory system.

In all allosamidin-producing strains we isolated, most of the allosamidin produced has been obtained from mycelia cultured in a chitin-free medium such as a Bennet medium [20]. To know the mechanism of allosamidin transfer among cells, its localization in the culture broth was investigated with the chitin medium. Strain AJ9463 was cultured in a Bennet medium to obtain the mycelia which contained allosamidin. When the mycelia were collected and shaken in an inorganic salts solution, most of the allosamidin was maintained in the mycelia after incubation. However, more than 80% of the allosamidin was recovered from the culture filtrate when the mycelia were incubated in the chitin medium. This indicated that chitin or some other factors such as degradation products of chitin could release allosamidin from the mycelia, which is very important for allosamidin's action as a signal molecule and may validate our above experiments in which allosamidin was added exogenously into the chitin medium. In the chitin medium, allosamidin production by strain AJ9463 starts at around 72 h of cultivation and reaches a few hundred nM in the culture filtrate after 120 h of cultivation.

From this study, we can image the following physiological role of allosamidin in its producing *Streptomyces* (Fig. 5). Since the growing phase of mycelia in *Streptomyces* is not synchronized, allosamidin production would start in some part of mycelia at the middle growing phase. The allosamidin produced would be released to the outside of the mycelia in the presence of chitin and transported to any part of mycelia in the allosamidin-producing strain itself or other allosamidin-producing strains. It would activate the chitinase production 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, which would further promote growth of the allosamidin-producing strains in a chitin-rich environment. This is the first model demonstrating that a microbial secondary metabolite with a specific biological activity shows clear physiological activity toward its producer. Study to confirm allosamidin's role in a real environment is now in progress.

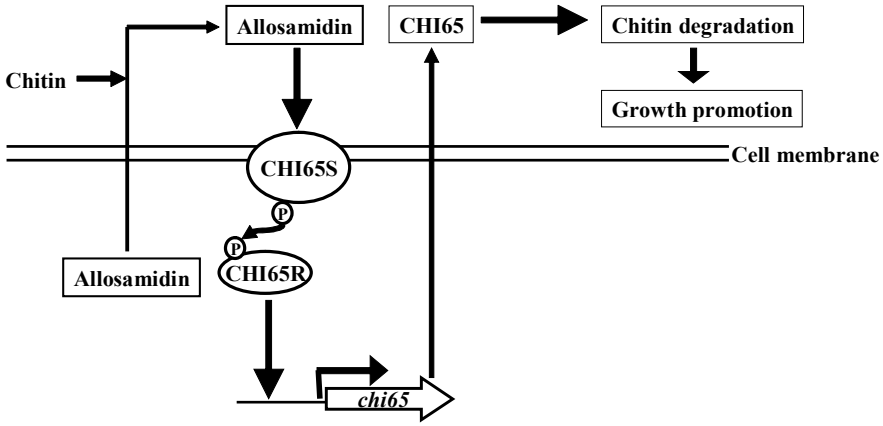

Figure 5 : Allosamidin is released from the microbial mycelia by responding to chitin and it strongly activates transcription of a gene (*chi65*) encoding a main chitinase secreted to the culture filtrate through a two-component regulatory system (CHI65S, a sensor histidine kinase, and CHI65R, a response regulator). This enhancement of chitinase production leads to promoting chitin degradation and microbial growth.

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