NgcE\textsuperscript{Sco} Acts as a Lower-Affinity Binding Protein of an ABC Transporter for the Uptake of N,N'-Diacyethylchitobiose in \textit{Streptomyces coelicolor} A3(2)

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In the model species \textit{Streptomyces coelicolor} A3(2), the uptake of chitin-degradation byproducts, mainly N,N'-diacyethylchitobiose ([GlcNAc]_2) and N-acetylgalactosamine (GlcNAc), is performed by the ATP-binding cassette (ABC) transporter DasABC-MsiK and the sugar-phosphotransferase system (PTS), respectively. Studies on the \textit{S. coelicolor} chromosome have suggested the occurrence of additional uptake systems of GlcNAc-related compounds, including the SCO6005-7 cluster, which is orthologous to the ABC transporter NgcEFG of \textit{S. olivaceoviridis}. However, despite conserved synteny between the clusters in \textit{S. coelicolor} and \textit{S. olivaceoviridis}, homology between them is low, with only 35% of residues being identical between NgcE proteins, suggesting different binding specificities. Isothermal titration calorimetry experiments revealed that recombinant NgcE\textsuperscript{Sco} interacts with GlcNAc and (GlcNAc)_2, with \(K_d\) values (1.15 and 1.53 \(\mu\)M, respectively) that were higher than those of NgcE of \textit{S. olivaceoviridis} (8.3 and 29 nM, respectively). The disruption of \textit{ngcE Sco} delayed (GlcNAc)_2 consumption, but did not affect GlcNAc consumption ability. The \textit{ngcE Sco}-\textit{dasA} double mutation severely decreased the ability to consume (GlcNAc)_2 and abolished the induction of chitinase production in the presence of (GlcNAc)_2, but did not affect the GlcNAc consumption rate. The results of these biochemical and reverse genetic analyses indicate that NgcE\textsuperscript{Sco} acts as (GlcNAc)_2-binding protein of the ABC transporter NgcEFG\textsuperscript{MsiK}. Transcriptional and biochemical analyses of gene regulation demonstrated that the \textit{ngcE Sco} gene was slightly induced by GlcNAc, (GlcNAc)_2, and chitin, but repressed by DasR. Therefore, a model was proposed for the induction of the chitinolytic system and import of (GlcNAc)_2, in which (GlcNAc)_2 generated from chitin by chitinase produced leakily, is mainly transported via NgcEFG-MsiK and induces the expression of chitinase genes and \textit{dasABCD}.

\textbf{Key words:} \(N,N\textsuperscript{'}-\text{diacetylchitobiose}, \text{ABC transporter}, \text{chitin, chitinase, DasR}\)

\textit{Streptomyces} are multicellular mycelial bacteria that thrive in soil environments as well as in marine and fresh water ecosystems. As producers of a large range of secondary metabolites, including two-thirds of all known antibiotics as well as many anticancer, antifungal and immunosuppressive agents, streptomycetes are of utmost importance for human health, agriculture, and biotechnology (1, 2). Streptomycetes have a saprophytic lifestyle and degrade all naturally occurring biopolymers; therefore, they are a rich source of industrially relevant enzymes (12, 47). These bacteria are major decomposers of chitin, a polymer of \(\beta\)-1,4-linked N-acetylmuramylglycosamine (GlcNAc) units. Complete chitin degradation into GlcNAc and \(N,N\textsuperscript{'}\text{-diacyethylchitobiose} ([\text{GlcNAc}]_2)\) by streptomycetes requires the production of extracellular chitinases of families 18 and 19 of the glycoside hydrolase (GH) classification (for a review, see [28]), intra- and extracellular N-acetyl-\(\beta\)-D-glucosaminidases of GH families 3 and 20 (15, 33, 44), and the lytic polysaccharide monoxygenase of AA10 (21), the amino acid sequence of which is similar to chitin-binding proteins (29, 38).

The uptake of chitin degradation byproducts was initially studied in \textit{Streptomyces olivaceoviridis}, which uses PtsC2, the transmembrane enzyme IIC of the phosphoenolpyruvate phosphotransferase system (PTS), and the ATP-binding cassette (ABC) transporter NgcEFG for GlcNAc uptake (30, 49, 51). NgcEFG also internalizes (GlcNAc)_2. \textit{S. coelicolor} A3(2) transports GlcNAc via the Pts enzyme IIC NagE2 as a potentially unique uptake system for GlcNAc when this nutrient is provided as the main carbon source (24), while the uptake of (GlcNAc)_2 is mediated by the ABC transporter DasABC (31) for subsequent hydrolysis into GlcNAc by the \(N\text{-acetyl-}\beta\)-D-glucosaminidase DasD (33). The catabolism of GlcNAc further requires the GlcNAc kinase NagK, GlcNAc-6-phosphate deacetylase NagA, and GlcN-6-P deaminase/isomerase NagB in order to generate fructose-6-phosphate, which will enter glycolysis (39). The expression of all \textit{pts}, \textit{nag}, and \textit{das} genes encoding GlcNAc and (GlcNAc)_2 transporters and catabolic enzymes is inhibited by the GntR family transcription factor DasR, the DNA-binding activity of which is repressed by GlcNAc-6-P and GlcN-6-P (6, 9, 22, 23, 27, 41, 43). The expression of all of these genes is activated by

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GlcNAc, except for the dasA gene, the transcription of which is induced by chitin and (GlcNAc)2 and repressed by GlcNAc (6, 31), similar to the genes encoding chitinase (chi) (20). DasR is required for the maximal expression of chi genes (22), while in the closely related actinobacterium Saccharopolyspora erythreae, DasR acts as a transcriptional repressor of chi genes, similar to other chitin/GlcNAc utilization genes (17).

The DasABC system uses the multiple sugar import protein MsIK as an ATPase (32). The inactivation of msIK abolishes (GlcNAc)2 consumption, whereas the dasA-null mutant maintains the ability to consume (GlcNAc)2, but at a markedly lower rate (31). These findings suggest that there is at least one additional ABC transporter for the uptake of (GlcNAc)2, which also involves MsIK as a common ATPase component (32). In S. coelicolor, the MsIK-mediated uptake of (GlcNAc)2 is required not only for the utilization of chitin degradation byproducts, but also to induce chitinase production (32). However, the inactivation of dasA resulted in stronger total chitinase activity by S. coelicolor, which is not consistent with a simple induction model that requires the transport of (GlcNAc)2 to trigger the chitinolytic system (6, 31). This phenotype suggests that the proper induction of chitin utilization genes needs to involve diverse sensory/transporter systems that act synergistically or competitively according to the extracellular concentration pattern of chitin-derived nutrients (6).

In order to improve our understanding of the chitin utilization system in streptomycetes, we investigated the role of the SCO6005-6007 gene cluster of S. coelicolor, which has a homologous gene organization and genomic context to the genes for the high-affinity GlcNAc and (GlcNAc)2 NgcEFG transporter of S. olivaceoviridis (30, 51). However, while gene synteny is conserved, similarities at the amino acid level between SCO6005-6007 gene products and NgcEFG were low for orthologous proteins. In the present study, we investigated how the lack of similarities between these orthologous transporters impacts on the capacity of the S. coelicolor NgcEFG (NgcEScos) system to consume and respond to GlcNAc and (GlcNAc)2 using biochemical and reverse-genetic analyses.

Materials and Methods

Bacterial strains, plasmids, and media
S. coelicolor A3(2) strain M145 (14), its dasA- and dasR-null mutants ASC2 and BAP29 (27, 31), and dasR-overexpressing strain carrying the multicopy dasR gene (dasR++) (26) were used. Escherichia coli JM109 (52) and DH5α (42) were used as hosts for gene manipulation. E. coli ET12567 (dam dcm hisD) (18) was used to prepare plasmids for S. coelicolor transformation in order to avoid the methylation-specific restriction system of the bacterium. E. coli BL21(DE3)pLysS (Novagen, Burlington, MA, USA) was used to overproduce the NgcEScos and DasR proteins. The plasmids used in the present study are listed in Table S1. Luria–Bertani (LB) medium (34) was used to culture S. coelicolor. E. coli transformants were grown in LB medium supplemented with 50 μg mL−1 ampicillin or 10 μg mL−1 gentamycin. Minimal medium (MM; 10 mM KH2PO4, 10 mM K2HPO4, 1 mM CaCl2, and 0.5 mM MgCl2 supplemented with 0.1% [v/v] trace element solution) (35) was used to investigate the responses of S. coelicolor cells to various carbon sources. Soya flour—mannotol (SFM) agar medium (14) was used to prepare spores of S. coelicolor strains.

Gene manipulation
Plasmid preparation and restriction enzyme digestion were performed as described by Sambrook & Russell (2001) (34). DNA fragments were ligated using a DNA ligation kit (Takara Bio, Kusatsu, Japan) according to the manufacturer’s instructions.

Production and purification of recombinant NgcEScos and NgcE proteins
Two sets of primers (Table S2) were designed to amplify parts of the SCO6005 (ngcEScos) gene, which encode the part of the NgcEScos protein without the putative signal peptide (29 amino acids from the N terminus). The recombinant NgcEScos protein was tagged with an N-terminal 6-His or N-terminal GST using pET16b or pGEX-4T-1 (Table S1). Both recombinant NgcEScos proteins were successfully overproduced in a soluble form and purified using Ni-NTA agarose (Qiagen, Hilden, Germany) and Glutathione Sepharose 4B (GE Healthcare, Waukesha, WI, USA), respectively. The recombinant N-terminally His-tagged NgcE protein of S. olivaceoviridis was also produced in E. coli carrying pQE301 (Table S1) and purified as reported previously (30). The purified His-tagged NgcEScos protein was used to prepare anti-NgcEScos antisera, while binding affinities for the sugars of the purified GST-tagged NgcEScos protein were assessed as described below following the removal of the GST-tag. The sugar-binding affinity of the purified His-tagged NgcE protein was also analyzed as described below. See the Supplementary Materials and Methods for detailed conditions pertaining to protein production and purification.

Isothermal titration calorimetry (ITC)
ITC experiments were performed with an iTC200 System (GE Healthcare) (50). Solutions were thoroughly degassed prior to experiments in order to avoid air bubbles in the calorimeter. A volume of 0.2028 mL of NgcEScos solution (19 μM) in 20 mM Tris/HCl buffer (pH 8.0) at 30°C was placed in the reaction cell, and ligand solutions in identical buffers were placed in the ITC syringe. In all titrations, 0.8-μL aliquots were injected into the reaction cell at 80-s intervals with a stirring speed of 1,000 rpm. Titrations were completed after 40 injections. The shape of the ITC binding curve was assessed by the Wiseman c value. When titration experiments were performed with c values from 10 to 100 (c = N·Kᵢ[M]; where N is stoichiometry, Kᵢ is the association constant, and [M] is the initial protein concentration), the Kᵢ values obtained were regarded as being reliable (50). ITC data were collected and fit automatically using microcal origin v. 7.0 software accompanying the iTC200 system (50). All data from the binding reactions fit well with the single-site binding model yielding stoichiometry (N), an equilibrium dissociation constant (Kᵢ), and enthalpy change (∆H). The reaction free energy change (∆G) and entropy change (∆S) were calculated from the relationship described in the following equation: ∆G = −RT ln (Kᵢ) − TAS.

Assessment of binding affinities for sugars based on alterations in fluorescent strength
The Kᵢ value of NgcEScos or NgcE was measured against N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannuramic acid, glucose, xylose, or mannose based on a fluorescence method (10).

Disruption of the ngcEScos gene
The ngcEScos gene was disrupted in the wild-type strain S. coelicolor A3(2) M145 and its dasA-null mutant ASC2 (31) by homologous recombination using the temperature-sensitive plasmid pAS100 (Table S1) (51). Most of the ngcEScos gene was replaced by the aacC4 gene cassette (Fig. S3 and S4) (3). Detailed methods are described in the Supplementary Materials and Methods.

Complementation of the ngcEScos gene cluster
As derivatives of the multi-copy plasmid vector pWHM3 (Table S1) (45), the plasmids pWHM3-ngcEFG and pWHM3-ngcFG were prepared to express ngcEFGScos and ngcFGScos, respectively, with the native promoter region (Fig. S3). Details for constructing these
plasmids are provided in the Supplementary Materials and Methods. These constructs were introduced into *S. coelicolor* strains via protoplast transformation (14).

**Conditions for the *S. coelicolor* culture**

In order to investigate the responses of cells to various sugars, we cultured *S. coelicolor* strains according to a previously described method (31). Spores formed on SFM agar medium were inoculated into 30 mL of LB medium in a 100-mL flask with a spring (14) and grown at 30°C for 18–20 h on a rotary shaker at 150 rpm. Mycelia were harvested by centrifugation (3,000 rpm, 3 min), washed in 60 mL of MM, and divided into several aliquots. Each aliquot was supplemented with a different carbon source: 250 μM of glucose, maltose, cellobiose, xylitol, glucosamine, GlcNAc, or (GlcNAc)₂ and 0.05% (w/v) colloidal chitin. After sugar supplementation, cultures were again grown at 30°C on a rotary shaker at 150 rpm. In measurements of GlcNAc and (GlcNAc)₂, concentration rates, the amount of mycelia in MM was adjusted to 19–21 mg fresh weight/mL culture⁻¹. Culture fluids were sampled periodically, centrifuged to separate the supernatant and mycelia, and stored at −80°C. The sugar concentrations and chitinase activities of the supernatants were measured, whereas mycelia were used for total RNA preparation and immunoblot analyses.

**Measurement of sugar concentrations**

GlcNAc and (GlcNAc)₂ concentrations were measured in culture supernatants using high-performance liquid chromatography with UV detection at 215 nm (SPD-20A; Shimadzu, Kyoto, Japan) and a gel electrophoresis in a 1% (w/v) agarose gel and the fluorescence was applied as previously described (43). Probes were separated by subsequent purification onto a Ni²⁺-nitrilotriacetic acid-agarose column (GE Healthcare). The sequences of the oligonucleotides used for EMSAs were performed using Cy5-labeled *dre* probes (final concentration, ~0.1 mM) and *DasR*-6His (final concentration, ~1 mM) in a total reaction volume of 50 μL. The protocol for *DasR*-6His production from pFT240 (Table S1) (26) in *E. coli* BL21(DE3) and subsequent purification onto a Ni²⁺-nitrilotriacetic acid-agarose column was applied as previously described (43). Probes were separated by gel electrophoresis in a 1% (w/v) agarose gel and the fluorescence of the probes was visualized using a Typhoon Trio + variable mode imager (GE Healthcare). The sequences of the oligonucleotides used to generate Cy5-fluorescent double-stranded DNA probes (dre<sub>extrOK</sub>, dre<sub>extrA</sub>, and dre<sub>extrE</sub>) are described in Table S2.

**Electromobility gel shift assays (EMSAs)**

EMSAs were performed using Cy5-labeled *dre* probes (final concentration, ~0.1 mM) and *DasR*-6His (final concentration, ~1 mM) in a total reaction volume of 50 μL. The protocol for *DasR*-6His production from pFT240 (Table S1) (26) in *E. coli* BL21(DE3) and subsequent purification onto a Ni²⁺-nitrilotriacetic acid-agarose column was applied as previously described (43). Probes were separated by gel electrophoresis in a 1% (w/v) agarose gel and the fluorescence of the probes was visualized using a Typhoon Trio + variable mode imager (GE Healthcare). The sequences of the oligonucleotides used to generate Cy5-fluorescent double-stranded DNA probes (dre<sub>extrOK</sub>, dre<sub>extrA</sub>, and dre<sub>extrE</sub>) are described in Table S2.

**ChIP-on-chip and microarray analysis**

ChIP-on-chip and microarray analyses of the *DasR* binding event on the *ngcE<sup>Eco</sup>* upstream region and the transcription profiles of *ngcE<sup>Eco</sup>* respectively, were retrieved from raw data published as supplementary files from Świątek-Połatyńska et al. (2015) (41).

**Reverse transcription-PCR**

DNA-free total RNA was prepared from mycelia using our method (31) and an SV Total RNA Isolation System (Promega, Madison, WI, USA). In order to characterize transcripts, a reverse transcription (RT)-PCR analysis was performed using AccuPower RT/PCR Premix (Bioneer, Daejeon, Korea) as reported previously (31). A set of primers specific for the *ngcE<sup>Eco</sup>* transcript was designed to give a PCR product of 540 bp (Table S2). In PCR, the number of cycles was set to 20 in order to avoid the saturation of PCR product formation. RT-PCR experiments without prior RT were performed in order to ensure that no residual DNA was present in the RNA samples.

In expression studies on *dasA*, *nagE2*, and *ngcE<sup>Eco</sup>* in *S. coelicolor* M145, the RNAs of the *dasR* null mutant (BAP29) and the strain overexpressing *dasR* (*dasR<sup>++)*) were collected after 30 h of growth in MM mannitol (0.5% [w/v]) agar plates with or with 1% GlcNAc. In the semi-quantitative analysis, samples were taken at four-cycle intervals between cycles 27–35 in order to compare non-saturated PCR product formation (amplifications at cycles 27 and 31 are presented in the first and second wells of each assay). RT-PCR without reverse transcription was performed as a control in order to confirm the absence of residual DNA. Data were verified by three independent experiments.

**Immunoblot analysis**

*S. coelicolor* mycelia, which were incubated for 4 h in MM supplemented with 250 μM of each carbon, were harvested by centrifugation (18,000×g, 4°C, 3 min), suspended in phosphate-buffered saline (34), and disrupted by sonication (15 s×8) on ice. The suspension was centrifuged at 10,000×g at 4°C for 5 min, and the protein concentration of the supernatant was measured by Bradford’s method (4). Proteins corresponding to 50 μg were separated with 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (16) and blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millpore, Burlington, MA, USA). *Anti-DasA* antiserum (31) and *anti-NgcE<sup>Eco</sup>* antiserum, which were prepared using the His-tagged NgcE<sup>Eco</sup> protein as an antigen, were used in the immunoblot analysis.

**Results**

**In silico analysis of SCO6005-6007 of *S. coelicolor***

SCO6005 encodes a putative extracellular sugar-binding component of the transporter (pfam01547), the orthologous protein of which in *S. lividans* is exported via the twin-arginine translocation (TAT) pathway (11). The gene cluster includes two additional ORFs encoding the putative ABC-type integral membrane proteins (SCO6006 and SCO6007) that form a transporter permease (Fig. S1). Regarding most streptomycetes sugar ABC transporters, the gene for the ATPase component was not included in the cluster and energy for sugar import was most likely provided by the multiple sugar import ATPase MsIK (13, 32, 36, 37, 46). The Rok family regulatory gene *rokB<sup>77</sup>* is immediately downstream of the operon, and controls the xylose operon SCO6009-6011 (40). Upstream of SCO6005, 6004 encodes a putative alpha-1,2-mannosidase.

The SCO6005-6007 operon of *S. coelicolor* is an orthologue of the *S. olivaceoviridis* NgcE<sup>FO</sup> operon, which encodes a high-affinity transporter for GlcNAc and (GlcNAc)₂ (30, 51). While gene synteny is strictly conserved in streptomycetes, identity at the amino acid level between *S. coelicolor* SCO6005-6007 gene products and NgcE<sup>FO</sup> is low for orthologous proteins, namely 35% amino acid identity for the SCO6005 protein and NgcE, 44% for SCO6006 and NgcF, and 50% between SCO6007 and NgcG (Fig. S1). In contrast, the other streptomycetes NgcE orthologues share between 80 to 91% amino acid identities throughout the full-length sequence. These low amino acid identities between *S. coelicolor* and *S. olivaceoviridis* and other streptomycetes are limited to the three Ngc proteins because the putative products of adjacent ORFs *SCO6004* and *SCO6008* (ROK<sup>77</sup>B<sup>77</sup>) present high levels of identity, as expected for orthologous proteins.
The lack of identity between Ngc proteins from *S. coelicolor* and *S. olivaceoviridis* prompted us to assess the binding affinity of the solute-binding component of the transporter of *S. coelicolor* (NgcESc). The binding specificity and affinity of the pure NgcESc protein heterologously produced in *E. coli* (see Materials and Methods for details) was initially investigated using ITC. As shown in Fig. S2, the quantity of heat of the NgcESc solution increased with the concentrations of GlcNAc and (GlcNAc)_2, but was not affected by the addition of (GlcNAc)_3 and higher oligomers up to (GlcNAc)_6, thereby demonstrating that the recombinant NgcESc protein interacted with GlcNAc and (GlcNAc)_2. NgcESc and GlcNAc/(GlcNAc)_2 bound in a 1:1 stoichiometry and binding in both cases was driven by entropy, while the loss of entropy opposed binding, suggesting a specific interaction between NgcESc and GlcNAc/ (GlcNAc)_2 (Table 1). Values were calculated based on increments in the fluorescent strength after the addition of an increasing amount of GlcNAc. The values of NgcESc for GlcNAc and (GlcNAc)_2 were 1.15 and 1.53 μM, respectively (Table 1). These values were higher than those of *S. olivaceoviridis* NgE for GlcNAc and (GlcNAc)_2 i.e. 8.3 and 29 nM, respectively (51), and that of DasA for (GlcNAc)_2 i.e., 32 nM (31).

In order to more precisely compare the affinity of NgcESc with that of NgE, the recombinant NgcESc and NgE proteins produced in *E. coli* were purified and their affinities were evaluated based on changes in the fluorescent strengths of the proteins. The addition of GlcNAc did not quench the fluorescent strengths of the proteins, it increased them. The values were calculated based on changes in the fluorescent strength after the addition of an increasing amount of GlcNAc. The *K_d* value of NgcESc for GlcNAc was 1.9 μM, which corresponded with that obtained by ITC (Table 1). The *K_d* value of NgcE produced in *E. coli* for GlcNAc was 85 nM. Although this value was one magnitude higher than that obtained by surface plasmon resonance, it is still 22-fold lower than that of NgcESc, indicating the markedly higher affinity of the NgcE protein. (GlcNAc)2 did not modify the fluorescence properties of NgcESc or NgE. The *K_d* values of NgcESc for N-acetylgalactosamine (GalNAc) and N-acetylmuramic acid (MurNAc) were 12 and 25 μM, respectively, and were 6- and 13-fold higher than that for GlcNAc (1.9 μM). We also investigated the effects of xylose and mannose on the fluorescent strength of NgcESc due to the presence of genes coding for putative mannosidase and a regulator of the xylose operon in the vicinity of the ngcEFG operon (Fig. S1). Glucose, xylose, or mannose up to 1 mM did not significantly affect the fluorescent strength of NgcESc, implying the absence of an interaction between NgcESc and these sugars. The *K_d* values of the maltose-binding protein (MBP), L-arabinose-binding protein (ABP), and D-glucose/D-galactose-binding protein (GBP) of ABC transporters for the corresponding ligand sugars range between 10⁻⁸ and 10⁻⁶ M (25). The *K_d* values of NgcESc for GlcNAc and (GlcNAc)_2 were in the 10⁻⁶ M range (Table 1), implying that the protein mediates the uptake of these sugars; however, affinities were lower than those of *S. olivaceoviridis* NgE for GlcNAc and (GlcNAc)_2.

**Table 1.** Thermodynamic parameters for N-acetylgalactosamine (GlcNAc) and N,N′-diacetylchitobiose ((GlcNAc)_2) binding to NgcESc obtained from ITC profiles shown in Fig. S2.

| Ligand     | N   | *K_d* (μM) | ΔΗ (kcal mol⁻¹) | ΔS (cal mol⁻¹ K⁻¹) | −TΔS (kcal mol⁻¹) | ΔG (kcal mol⁻¹) |
|------------|-----|------------|----------------|-------------------|-----------------|----------------|
| GlcNAc     | 1.05| 1.15       | −9.98          | −5.77             | 1.75            | −8.23          |
| (GlcNAc)_2 | 0.98| 1.53       | −12.3          | −14.0             | 4.24            | −8.06          |

N, binding stoichiometry; *K_d*, dissociation constant; ΔΗ, change in enthalpy; ΔS, change in entropy; T, temperature; ΔG, change in Gibbs free energy.

The ngcESc gene was disrupted in *S. coelicolor* strain M145 and its *dasA* null-mutant ASC2 (Fig. S3 and S4) in order to assess its contribution to GlcNAc and/or (GlcNAc)_2 uptake. The mycelia of strains M145, ASC2, the ngcESc-null mutant (strain CI1), and *dasA*-ngcESc double-null mutant (strain CI3), pregrown in LB medium, were cultivated in MM supplemented with 250 μM of GlcNAc or (GlcNAc)_2. GlcNAc consumption rates were not significantly affected by the disruption of ngcESc regardless of whether they were examined in the wild-type or *dasA*-minus background (Fig. 1A). The disruption of msiK lowered the rate of GlcNAc consumption (Fig. 1A), suggesting the presence of ABC transporter(s) for GlcNAc uptake.

In contrast to GlcNAc, the (GlcNAc)_2 consumption pattern was affected by the ngcESc mutation (Fig. 1B). (GlcNAc)_2 consumption in the wild-type strain M145 was divided into two steps: the initial step from 0–2 h (2.3 nmol h⁻¹ mg mycelia⁻¹ [R²=0.991]) and the next induced step from 2–3 h (6.0 nmol h⁻¹ mg mycelia⁻¹ [R²=0.998]). In the ngcESc-null mutant, (GlcNAc)_2 consumption was delayed (Fig. 1B). In the first step during 0–2 h, the (GlcNAc)_2 concentration remained almost constant, and the initiation of the next induced consumption was delayed for 0.5–1 h. The consumption rate of induced consumption (3–4 h) in CI1 was 8.6 nmol h⁻¹ mg mycelia⁻¹ (R²=0.993), as reported previously (31). In the *dasA*-minus background, the effects of the disruption of ngcESc were more obvious. The *dasA*-ngcESc double mutant CI3 showed a low level of (GlcNAc)_2 consumption (1.2 nmol h⁻¹ mg mycelia⁻¹ [2–5 h, R²=0.986]). The msiK-null mutant ASC2 consumed (GlcNAc)_2 constantly (2.8 nmol h⁻¹ mg mycelia⁻¹ [0–5 h, R²=0.993]), as reported previously (31).

**Chitinase production in the ngcESc mutant**

We previously reported that (GlcNAc)_2 uptake is necessary for the induction of chitinase production in *S. coelicolor* (32).
In order to elucidate the involvement of NgcE Sco in chitinase production, the effects of the disruption of ngcE Sco on chitinase production were investigated. As shown in Fig. 2A, the chromosomal deletion of ngcE Sco reduced the level of chitinase activity induced in the presence of (GlcNAc)2. In contrast, the dasA-ngcE Sco double mutant fully abolished chitinase production in the presence of (GlcNAc)2 (Fig. 2A), as observed for the msiK-null mutant ASC3 (32). The amounts of mycelia in MM (mg wet weight mL−1) were as follows: 21 (M145), 21 (CI1), 19 (ASC2), 20 (CI3), and 21 (ASC3). If necessary, consumption rates were calculated based on the concentrations of the corresponding sugars during the periods in which a linear decline in sugar concentrations was observed.

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Similar to the dasA mutant (Fig. 2A), the induced level of chitinase activity was markedly higher in strain CI3, which carries the ngcEFGsco operon on a multi-copy plasmid (pWHM3-ngcEFG), than in CI1, which is the ngcE-F-sco mutant carrying the empty vector (pWHM3) (Fig. 2C). The production of NgcE Sco in complemented strain CI3 (pWHM3-ngcEFG) was confirmed by the immunoblot analysis using anti-NgcE Sco antiserum (Fig. S5).

In order to elucidate the roles of the distinctive transporters in the induction of the chitinolytic system, we assessed chitinase production profiles in the presence of lower concentrations of (GlcNAc)₂, the level of chitinase activity in the CI3 mutant CI3 (pWHM3-ngcE Sco) was similar to that in the presence of 50 μM (GlcNAc)₂ in M145 and CI3, while the ngcE-sco mutant and the parental strain M145 exhibited very weak chitinase activities at this concentration (Fig. 2D).

**Regulation of ngcE Sco expression**

A ChIP-on-chip approach for S. coelicolor M145 carrying the integrative vector pGAM29, which expresses C-terminally 3×FLAG-tagged DasR (see [41] for details), revealed DasR binding to the intergenic region between SCO6004 and SCO6005 (ngcE Sco) (Fig. 3A). This region possesses the predicted DasR responsive element (dre) ATGGGACTATACCTGT at nt position ~334 upstream of SCO6005 (drengcE) (Fig. 3A), which matches 12 out of the 16 nt of the dre consensus sequence (5). The DasR-binding event was abolished when S. coelicolor was grown in the presence of GlcNAc (Fig. 3A). In order to confirm ChIP-on-chip data, EMSAs were performed using pure His-tagged DasR (DasR-6His) and a short double-stranded oligonucleotide centered on drengcE (Table S2). DasR interacted with the DNA probe containing drengcE, as observed with the positive control probes containing dre upstream of nagK4 and dasA (drengK4 and dreasA) (Fig. 3B). The binding of DasR to the drengcE-containing probe was inhibited by GlcNAc-6P and GlcN-6P (Fig. 3C). GlcNAc-6P inhibited binding more efficiently than GlcN-6P (Fig. 3C). These results of the ChIP-on-chip analysis and EMSAs were consistent with those reported for the interactions of DasR with other dre genes (41, 43). GlcNAc-derived GlcNAc-6P and GlcN-6P inhibited the binding of DasR to dre in the ChIP-on-chip analysis.

Previous transcriptomic studies also revealed that ngcE Sco expression was induced by chitin (23) and GlcNAc (41). The microarray analysis revealed that the expression of ngcE Sco was up-regulated in the dasR mutant in the absence of GlcNAc and appeared to be induced at earlier time points (24 and 30 h) when S. coelicolor M145 was grown in MM medium supplemented with GlcNAc (Fig. S6A). Very similar expression profiles were observed for ngcE Sco and ngcG Sco (41), suggesting that ngcE Sco (SCO6005), ngcE Sco (SCO6006), and ngcG Sco (SCO6007) form a tri-cistronic operon that was herein confirmed using the RT-PCR analysis (Fig. S6B). In the dasR mutant, ngcE Sco transcription was not induced by GlcNAc (Fig. S6A). When mycelia grown in LB were exposed to 250 μM glucose, maltose, cellobiose, xylose, GlcNAc, or (GlcNAc)₂, the amounts of ngcE Sco transcripts were similar among the tested conditions (Fig. S6C), whereas dasA transcription was strongly induced in the presence of (GlcNAc)₂ under the same culture conditions (31, 32). In order to investigate the expression of ngcE Sco at the level of protein production, an immunoblot analysis was performed using antibodies against the recombinant His-tagged NgcE Sco protein overproduced in E. coli. NgcE Sco production was observed in the presence of glucose, maltose, cellobiose, (GlcN)₂, GlcNAc, or (GlcNAc)₂ (Fig. 4A). The levels of production in the presence of GlcNAc and (GlcNAc)₂ were 1.3- and 1.4-fold higher than that in the presence of glucose, respectively. In contrast, DasA production was markedly induced by (GlcNAc)₂ and by the glucosamine dimer (GlcN)₂, though to a markedly lower degree (Fig. 4A). Since the abundant carbon and nitrogen sources contained in LB medium may affect ngcE Sco transcriptional responses to

**Fig. 3.** DasR interaction with the dre upstream of ngcE Sco. (A) ChIP-on-chip experiment showing the DasR-binding event upstream of SCO6005 (ngcE Sco). Plots show DasR binding in the wild-type grown in minimal medium (MM) mannitol (closed circles) and in MM mannitol with GlcNAc (open circles). Note the absence of a binding event when GlcNAc is added to the medium. Arrows indicate the orientation of the genes adjacent to the DasR-binding signal. (B) EMSA with pure DasR and Cy5 probes centered on dre upstream of ngcE Sco, and dasA, and nagK used as positive controls (ctrl +). The probe with the binding site of Cbp (7) was used as a negative control (ctrl -). (C) EMSA showing the allosteric effects of both GlcNAc-6P and GlcN-6P on DasR binding to drengcE. The concentrations of both ligands are expressed in mM. In B and C, the final concentration of DasR was set to ~1 mM.
amino sugars, we repeated the expression studies on RNA samples that were prepared from mycelia grown on MM mannitol (0.5% [w/v]) with or without GlcNAc (1.0% [w/v]) at 28°C for 30 h. Under these conditions, the transcription of ngcE Sco was stronger in the dasR mutant and weaker in the dasR++ strain than in the parental strain M145, demonstrating that DasR acts as a transcriptional repressor of ngcE Sco under these conditions (Fig. 4B). Similar to that observed for nagE2, the transcription of ngcE Sco was induced when GlcNAc was added to MM mannitol in the wild-type or dasR mutant, whereas the dasR++ strain was leaky and strongly induced by (GlcNAc)2 (Fig. 4B and S6B). The initial (GlcNAc)2 consumption rate in M145 (2.3 nmol h⁻¹ mg mycelia⁻¹) corresponded well with the constant (GlcNAc)2 consumption rate (2.8 nmol h⁻¹ mg mycelia⁻¹) in its dasA mutant, whereas the dasA-ngcE Sco and msiK mutants had markedly lower rates (1.2 and 0.6 nmol h⁻¹ mg mycelia⁻¹, respectively) (Fig. 1B). Therefore, we suggest that NgcE Sco acts as the constitutive sugar-binding protein of the ABC transporter NgeEFGSco-MsiK for the uptake of (GlcNAc)2 in S. coelicolor A3(2), while DasABC-MsiK is the main (GlcNAc)2 uptake system, the production of which is strongly induced by (GlcNAc)2. When consumption experiments were performed with various amounts of mycelia (5–15 mg mycelia mL⁻¹), the effects of the ngcE Sco mutation on (GlcNAc)2 consumption were negligible, in contrast to the disruption of dasA, which markedly reduced the (GlcNAc)2 consumption rate (data not shown), possibly reflecting the 50-fold higher Kᵣ value of NgcE Sco for (GlcNAc)2 than that of DasA. We assumed that remaining (GlcNAc)2 consumption in the dasA-ngcE Sco and msiK mutants was due to (GlcNAc)2 hydrolysis based on the basal level of extracellular N-acetylhexosaminidases and subsequent consumption of GlcNAc.

The reverse-genetic analysis did not indicate the involvement of ngcE Sco in the uptake of GlcNAc. The NgeEFGSco-MsiK system may not uptake GlcNAc even though NgcE Sco interacts with GlcNAc. The MalE protein, which is the maltose (maltodextrin)-binding protein for the uptake of maltose and maltodextrin in E. coli, interacts with ligands and mediates the uptake of sugars. Reduced or oxidized maltodextrins were not transported into cells, but bound to MalE with good affinity (8). Similarly, the “maltodextrin-negative” mutants of MalE only show a marginal decrease in affinity toward maltodextrins, but do not support the transport of maltodextrins in whole cells (48).

In the present study, we observed a reduced GlcNAc consumption rate in ASC3 (Fig. 1A) that lacks the msiK gene encoding the common ATPase component for sugar ABC transporters (32). These results imply the presence of ABC transporters for GlcNAc; however, a previous study reported that the NagE2 of PTS may be a unique permease mediating the uptake of GlcNAc in S. coelicolor (24).

The presence of higher (DasABC) and lower (NgeEFGSco) affinity uptake systems for GlcNAc, in S. coelicolor, is likely to have a biological meaning. Similarly, in S. olivaceoviridis, the uptake of GlcNAc is mediated by two systems, the affinities of which are distinctive: the Kᵣ value of one system (the PTS system including PtsC2) for 14C-labeled GlcNAc is 5 μM, while that of the other system (ABC transporter containing NgeEFG) is 0.48 μM (30, 49).

The ngcE Sco, dasA double mutation abolished the induction of chitinase production by (GlcNAc)2 as the msiK mutation (Fig. 2A). These results clearly demonstrated that the uptake identity between the two orthologues is reflected in the Kᵣ values of NgcE Sco measured for GlcNAc and (GlcNAc)2 (1.15 and 1.53 μM, respectively [Table 1]), which were higher than those of the S. olivaceoviridis NgcE protein for GlcNAc and (GlcNAc)2 (8.3 and 29 nM, respectively) (51), and the Kᵣ value of DasA for (GlcNAc)2 (32 nM) (31). The expression of ngcE Sco was constitutive and induced to some extent by GlcNAc and (GlcNAc)2, while dasA expression was leaky and strongly induced by (GlcNAc)2 (Fig. 4B and S6B). The initial (GlcNAc)2 consumption rate in M145 (2.3 nmol h⁻¹ mg mycelia⁻¹) corresponded well with the constant (GlcNAc)2 consumption rate (2.8 nmol h⁻¹ mg mycelia⁻¹) in its dasA mutant, whereas the dasA-ngcE Sco and msiK mutants had markedly lower rates (1.2 and 0.6 nmol h⁻¹ mg mycelia⁻¹, respectively) (Fig. 1B). Therefore, we suggest that NgcE Sco acts as the constitutive sugar-binding protein of the ABC transporter NgeEFGSco-MsiK for the uptake of (GlcNAc)2 in S. coelicolor A3(2), while DasABC-MsiK is the main (GlcNAc)2 uptake system, the production of which is strongly induced by (GlcNAc)2. When consumption experiments were performed with various amounts of mycelia (5–15 mg mycelia mL⁻¹), the effects of the ngcE Sco mutation on (GlcNAc)2 consumption were negligible, in contrast to the disruption of dasA, which markedly reduced the (GlcNAc)2 consumption rate (data not shown), possibly reflecting the 50-fold higher Kᵣ value of NgcE Sco for (GlcNAc)2 than that of DasA. We assumed that remaining (GlcNAc)2 consumption in the dasA-ngcE Sco and msiK mutants was due to (GlcNAc)2 hydrolysis based on the basal level of extracellular N-acetylhexosaminidases and subsequent consumption of GlcNAc.

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The ngcE Sco, dasA double mutation abolished the induction of chitinase production by (GlcNAc)2 as the msiK mutation (Fig. 2A). These results clearly demonstrated that the uptake

Discussion

In the present study, we investigated the role of the ngcE Sco gene (SCO6005) and its encoding protein NgcE Sco in order to assess its contribution to the uptake and catabolism of chitin and its main byproducts GlcNAc and (GlcNAc)2. As discussed in the Introduction, we were unable to strictly refer to a previous study performed on ngcE in S. olivaceoviridis because despite the conserved synteny, the level of identity with NgcE Sco was only 35% (Fig. S1). The lack of amino acid

Fig. 4. Expression control of ngcE Sco in Streptomyces coelicolor A3(2). (A) The NgcE Sco protein produced in the presence of different mono- and disaccharides. The NgcE Sco protein was detected in the cell lysate of S. coelicolor A3(2) mycelia using anti-NgcE Sco antiserum. The DasA protein was also detected using anti-DasA antiserum for comparisons (31). Abbreviations: Glc, glucose; Mal, maltose; Cel, cellobiose; Xyl, xylobiose; GlcNAc, N-acetylglucosamine; (GlcNAc)2, N,N′-diacetylchitobiose; (GlcN)2, chitobiose. (B) Role of DasR and GlcNAc in the expression of ngcE Sco by semi-quantitative RT-PCR. The transcription of the GlcNAc-specific PTS EIIC component nagE2 (SCO2907) or dasA (SCO5232) was used as a positive control for DasR-dependent and GlcNAc-induced or -repressed genes, respectively. The transcription of 16S rRNA was used as a control for the DasR-independent gene (not shown). RNA samples were collected from S. coelicolor M145 (WT, wild-type), the dasR mutant BAP29 (∆dasR), and the dasR multicopy mutant (dasR++) grown at 28°C for 30 h (early transition phase) on MM mannitol agar plates with or without 1% GlcNAc. In the semi-quantitative analysis, samples were taken at four-cycle intervals in order to compare non-saturated PCR product formation (amplifications at cycles 27 and 31 are presented in the first and second wells for each assay). Data were verified in three independent experiments. See Table S1 for the oligonucleotides used.

The presence of higher (DasABC) and lower (NgcEFGSco) affinity uptake systems for GlcNAc, in S. coelicolor, is likely to have a biological meaning. Similarly, in S. olivaceoviridis, the uptake of GlcNAc is mediated by two systems, the affinities of which are distinctive: the Kᵣ value of one system (the PTS system including PtsC2) for 14C-labeled GlcNAc is 5 μM, while that of the other system (ABC transporter containing NgeEFG) is 0.48 μM (30, 49).

The ngcE Sco, dasA double mutation abolished the induction of chitinase production by (GlcNAc)2 as the msiK mutation (Fig. 2A). These results clearly demonstrated that the uptake
of (GlcN)\(_2\) is essential for the induction of chitinase production, as concluded in our previous study (32). It was noteworthy that the single nge\(^{E Sco}\) and dasA mutants exerted contrasting effects on the induction of chitinase production. The disruption of nge\(^{E Sco}\) reduced the chitinase activity induced by (GlcN)\(_2\), while the dasA mutation increased not only the levels of induced chitinase activity in the presence of (GlcN)\(_2\), or colloidal chitin, but also sensitivity to (GlcN)\(_2\) (Fig. 2A, B, and D). This result implies distinct roles for the two (GlcN)\(_2\) transporters. We assume that DasABC acts in the metabolism of (GlcN)\(_2\). The structures of the nge\(^{E Sco}\) and dasABC gene clusters imply roles for the encoding ABC transporters for (GlcN)\(_2\) uptake; a gene for the N-acetylglucosaminidase DasD hydrolyzing (GlcN)\(_2\) to GlcN is present in the dasABC gene cluster, whereas such a gene involved in (GlcN)\(_2\) hydrolysis is not clustered with nge\(^{E Sco}\) (Fig. S1). The disruption of dasD increased the level of chitinase production in the presence of (GlcN)\(_2\) or chitin (33). The dasD mutation may prolong the life of intracellular (GlcN)\(_2\), which induces chitinase production (33). We assumed that the higher sensitivity of the dasA mutant to (GlcN)\(_2\) in chitinase production (Fig. 2D) is attributed to the longer life of intracellular (GlcN)\(_2\), which induces the expression of chi genes. In contrast, the reduction in chitinase activity induced by (GlcN)\(_2\) in the nge\(^{E Sco}\) mutant may be ascribed to the shorter life of the disaccharide.

Nge\(^{E Sco}\) did not appear to be essential for the uptake of (GlcN)\(_2\) or induction of chitinase production (Fig. 1B, 2A, and B). However, it is involved in these processes and may have roles in the initial accumulation of intracellular (GlcN)\(_2\) for sensing chitin as a nutrient source in the environment. This hypothesis is supported by the observed late induction of chitinase production in the presence of colloidal chitin and the low initial (GlcN)\(_2\) consumption rate in the nge\(^{E Sco}\) mutant (Fig. 1B and 2B). In the presence of chitin, (GlcN)\(_2\) is expected to be continuously generated by chitin hydrolysis with extracellular chitinases produced leakily (or possibly by the chitinases of other microorganisms in ecosystems), and continually taken up mainly via NgeEFG-MsiK (Fig. 4B) until the (GlcN)\(_2\) concentration becomes sufficient to trigger the expression of das and chi (Fig. 5). Therefore, the intracellular accumulation of (GlcN)\(_2\) and subsequent induction of chitinase production may be delayed in the nge\(^{E Sco}\) mutant in the presence of colloidal chitin.

In the nge\(^{E Sco}\)-dasA mutant, the induction of chitinase production by colloidal chitin was markedly delayed (Fig. 2B). Chitinase production in the presence of colloidal chitin was abolished in the msiK mutant (Fig. 2B) (32), which implies the presence of additional ABC transporters for (GlcN)\(_2\) or the heterologous disaccharide GlcN-GlcN and/or GlcN-GlcN, which may be produced by the hydrolysis of colloidal chitin.

The results of RT-PCR, immunoblot assays, and previous transcriptomic and ChIP-on-chip analyses indicate that the expression of nge\(^{E Sco}\) is repressed by DasR and induced by GlcN, (GlcN)\(_2\), and chitin (22), though with a markedly weaker induction response to these elicitors than dasA in the presence of (GlcN)\(_2\) and nagE2 by GlcN (Fig. 3 and 4). It is noteworthy that the control of nge\(^{E Sco}\) expression is unique because it is the only known DasR-controlled gene

**Fig. 5.** Model of (GlcN)\(_2\) uptake and induction of das and chi genes in *S. coelicolor* A3(2). (Left part) In the presence of chitin, (GlcN)\(_2\) is continuously generated from chitin by extracellular chitinases produced leakily (or chitinases from other microorganisms in ecosystems), and is promptly taken up via NgeEFG-MsiK and, to a lesser extent, by DasABC-MsiK. This initial uptake unlocks the expression of das and chi genes. (GlcN)\(_2\) is partially hydrolyzed to GlcN by DasD and possibly other intracellular N-acetylglucosaminidases (GlcNases), which are leakily produced. (Right part) The induced chitinases increase the hydrolysis of chitin in order to generate larger amounts of (GlcN)\(_2\), which is mainly taken up by DasABC, the expression of which is induced by (GlcN)\(_2\). Imported (GlcN)\(_2\) is hydrolyzed to GlcN by DasD and other GlcNases. GlcN is converted to N-acetylglucosamine-6-phosphate (GlcN-6P) and glucosamine-6-phosphate (GlcN-6P) for its metabolism. GlcN-6P and GlcN-6P both interact with DasR in order to release the protein from the dre elements, thereby inducing the genes, including those for GlcN metabolism.
that is induced by GlcNAc, (GlcNAc)$_2$, and chitin. The in vivo binding pattern of DasR to dre$^{wei}$ differed from the patterns of the genes for DasA, chitinases, and GlcNAc metabolism. DasR binding to dre$^{wei}$ was inhibited by the presence of GlcNAc in MM, whereas DasR bound to the dre of DasA and chitinase genes (chiA, C, D, H, I, and J) (41). In R5 (nutrient rich) medium, DasR binding to the dre of the GlcNAc metabolic genes nagE2 and nagK4 was inhibited in the presence of GlcNAc, whereas DasR remained bound to dre$^{wei}$ (41). Although we concluded that NagE$^{ Sco }$ acted as a component of the ABC transporter for (GlcNAc)$_2$ in the present study, other physiological roles need to be investigated and elucidated.

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