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The permease gene *nagE2* is the key to *N*-acetylglucosamine sensing and utilization in *Streptomyces coelicolor* and is subject to multi-level control

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Summary

The availability of nutrients is a major determinant for the timing of morphogenesis and antibiotic production in the soil-dwelling bacterium *Streptomyces coelicolor*. Here we show that *N*-acetylglucosamine transport, the first step of an important nutrient signalling cascade, is mediated by the NagE2 permease of the phosphotransferase system, and that the activity of this permease is linked to nutritional control of development and antibiotic production. The permease serves as a high-affinity transporter for *N*-acetylglucosamine (K_m of 2.6 μM). The permease complex was reconstituted with individually purified components. This showed that uptake of *N*-acetylglucosamine requires a phosphoryl group transfer from phosphoenolpyruvate via the phosphotransferases EI, HPr and IIA^{Crr} to NagF, which in turn phosphorylates *N*-acetylglucosamine during transport. Transcription of the *nagF* and *nagE2* genes is induced by *N*-acetylglucosamine. Nutrient signalling by *N*-acetylglucosamine that triggers the onset of development was abolished in the *nagE2* and *nagF* mutants. *nagE2* is subject to multi-level control by the global transcription factor DasR and the activator AtrA that also stimulates genes for antibiotic actinorhodin biosynthesis. Hence, it is apparent that streptomycetes tightly control the nutritional state in a complex manner to ensure the correct timing for the developmental programme.

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Introduction

Streptomyces coelicolor A3(2) is a model organism for filamentous, soil-dwelling bacteria, which are main producers of beneficial compounds (antibiotics), and which undergo a complex living cycle from vegetative-growing hyphae to aerial hyphae and eventually spores (Chater, 1998; Flardh and Buttner, 2009). Antibiotic production and sporulation are initiated under adverse conditions such as nutrient limitation, for which extensive genetic re-programming is required (Claessen *et al.*, 2006; Hopwood, 2007). Mutants arrested in development are classified into two main categories, *bld* (bald, deficient in the formation of aerial hyhae) and *whi* (white, deficient in formation of grey-pigmented spores).

The focus of our research is to uncover the molecular mechanisms of nutrient sensing, transport and metabolism, which are directly linked with the decision to enter the developmental programme. A central system in bacteria that is involved in the uptake of numerous carbon sources and in carbon signalling is the phoephoenolpyruvate-dependent phosphotransferase system (PTS) (Brückner and Titgemeyer, 2002). During PTS-mediated carbon source uptake, a phosphoryl group is transferred from phoephoenolpyruvate to the general phosphotransferase enzyme I (EI), from there to HPr and further to the IIA domain of the permease complex. The latter is composed of three protein domains IIABC. Within the complex the phosphoryl group moves further to the IIB subunit, which eventually phosphorylates the carbon source entering through the transport channel. The PTS has been studied since more than 40 years ago mainly in

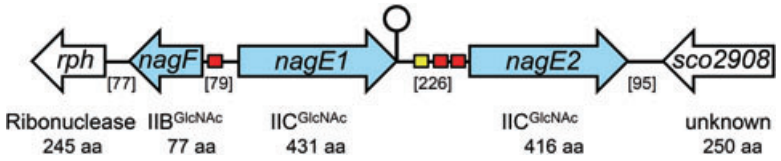

Fig. 1. Map of the *S. coelicolor nagF-nagE1-nagE2* (*sco2905–2907*) gene locus. Coding regions are indicated by arrows. Sizes of intergenic regions are shown by numbers of nucleotides in brackets. The predicted gene products are shown at the bottom. The positions of *cis*-acting elements are shown as three red boxes for DasR binding sites (*nagF*, ACTGGTCTACACCAGT; *nagE2*, ACAGGTCTACACCACT-x-AGTGGTGTAGACCACC) and one yellow box for AtrA binding site (GGAATCACGGGTTCC). A putative terminator structure is indicated by a loop sign (CGAAGGCCCCCGGACCGTCGGTCCGGGGGCCTTCT).

Escherichia coli and *Bacillus subtilis*, giving a profound molecular picture up to the level of the three-dimensional structures of interacting PTS proteins (Peterkofsky *et al.*, 2001). In all PTSs studied so far the stoichiometry of the phosphotransferases is monomeric for HPr, while all other exist as dimers (Boer *et al.*, 1994; Peterkofsky *et al.*, 2001; Fernández-Ballester *et al.*, 2003; Hurtado-Gómez *et al.*, 2006).

We have recently reported that nutrient signals of the PTS in *S. coelicolor* are inextricable linked to the onset of antibiotic production and morphogenesis (Rigali *et al.*, 2006; 2008). Inactivation of any of the three general phosphotransferases EI (encoded by *ptsI*), HPr (*ptsH*) and IIA^{Crr} (*crr*) of the PTS renders a non-sporulating (*bld*) phenotype. These *pts* mutants do not grow on *N*-acetylglucosamine (GlcNAc), most likely because EI, HPr and IIA^{Crr} are required to phosphorylate *N*-acetylglucosamine-specific PTS permease(s) by phosphoenolpyruvate-dependent phosphorylation (Nothaft *et al.*, 2003a). But the bald phenotype does not depend on the presence or absence of this aminosugar. We recently described a complete signalling pathway from sensing *N*-acetylglucosamine to the onset of antibiotic production, whereby the GntR-family transcriptional regulator DasR controls the genes for *N*-acetylglucosamine transport and metabolism and for antibiotic synthesis (Rigali *et al.*, 2008; van Wezel *et al.*, 2009).

N-acetylglucosamine is the preferred PTS substrate and induces the expression of EI, HPr and IIA^{Crr}, a homologue of *E. coli* enzyme IIA^{Glc} that serves in global carbon regulation (Nothaft *et al.*, 2003a,b). However, the GlcNAc uptake system of *S. coelicolor* was not identified in these studies. The distantly related *Streptomyces olivaceoviridis* has two *N*-acetylglucosamine transport systems, one of the PTS-type (PtsC2) and one of the ABC-type (ATP-binding cassette, NgcEFG) (Wang *et al.*, 2002; Saito and Schrempf, 2004). While the PTS is specific for *N*-acetylglucosamine, the ABC porter recognizes both *N*-acetylglucosamine and its disaccharide form chitobiose (*N,N'*-diacetylchitobiose [$(GlcNAc)_2$]) with similar affinities (Saito and Schrempf, 2004). *In silico* analysis of the *S. coelicolor* genome led to the identification of genes that encode at least six further PTS proteins, namely *nagE1*, *nagE2*, *malX1*, *malX2*, *ptx* and *sgaT* (Parche *et al.*, 2000). For the *malX2-nagE-nagE2* gene cluster (SCO2905-2907), it was predicted that it might encode *N*-acetyglucosamine-specific enzyme II permease proteins. This is corroborated by the characterization of the IIA^{Crr} protein that could serve as the IIA subunit of an *N*-acetylglucosamine-specific PTS in *S. coelicolor* (Kamionka *et al.*, 2002; Nothaft *et al.*, 2003a).

Here we present a molecular dissection of *N*-acetylglucosamine uptake in the model streptomycetes *S. coelicolor* A3(2), including the complete reconstitution of the PTS consisting of HPr, EI, IIA^{Crr}, IIB^{GlcNAc} and IIC^{GlcNAc}. This permease complex is solely responsible for the important nutrient and signalling molecule *N*-acetylglucosamine. We further demonstrate that transcriptional control of the GlcNAc transporter gene *nagE2* is mediated through the transcription factors DasR and AtrA that also play a crucial role in the control of antibiotic production, further underlining the intricate relationship between GlcNAc metabolism and antibiotic production.

Results

NagE2 is the major permease for N*-acetylglucosamine in* S. coelicolor

The *malX2-nagE1-nagE2* (SCO2905-2907) locus encoding putative *N*-acetylglucosamine-specific PTS permease proteins is depicted in Fig. 1. Considering that *malX2* encodes the enzyme IIB component of the GlcNAc-specific PTS permease (see below), we renamed it *nagF*. Since *nagE1* and *nagE2* were candidates to comprise the predicted integral membrane part of the transport channel, we generated the single mutants BAP4 (*nagE1::aacC4*) and BAP5 (*nagE2::aacC4*), and the double mutant BAP6 (*nagE1::aacC4::nagE2*). All the mutant strains developed normally on SFM and R2YE agar plates. To analyse possible effects in carbon utilization, we monitored growth in liquid mineral medium supplemented with various carbon sources. Interestingly, this showed that BAP5 and BAP6 were unable to grow on *N*-acetylglucosamine, while growth on glucose, fructose, xylose, mannose, sucrose or galactose was not impaired.

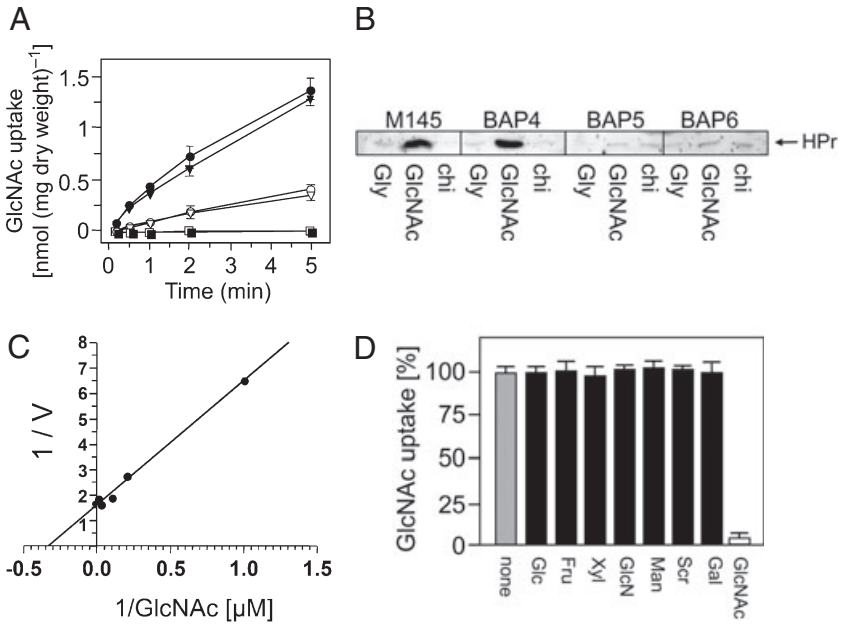

Fig. 2. A. Time-course experiment of GlcNAc uptake. GlcNAc uptake was measured for M145 and its mutant derivatives BAP4, BAP5 and BAP6. Mycelia were grown in mineral medium supplemented with glycerol, or glycerol plus GlcNAc. Strains are denoted as follows: M145 gly (○); M145 GlcNAc (●); BAP4 gly (▽); BAP4 GlcNAc (▼); BAP5 and BAP6 gly (□); BAP5 and BAP6 GlcNAc (■). Standard deviations given by error bars indicate the mean of triplicate measurements from three independent conducted experiments.
B. Detection of HPr by Western blot. Protein levels of HPr in crude cell extracts of M145, BAP4, BAP5 and BAP6 grown in the presence of either 50 mM glycerol, 50 mM GlcNAc or 1% chitin (the multimeric form of GlcNAc) are depicted. Note that induction of HPr by GlcNAc is completely lost in the absence of the transporter NagE2 (mutants BAP5 and BAP6).
C. Lineweaver-Burk plot. The graph displays the determination of the K_m and V_{max} of GlcNAc uptake of M145. Values were determined in triplicate at the following GlcNAc concentrations: 1, 5, 10, 50, 100, 150, 400 µM. The experiment was repeated twice.
D. Substrate specificity of NagE2. Mycelia of *S. coelicolor* wild type grown in mineral medium supplemented with 0.1% CAA plus 50 mM GlcNAc was incubated with 20 µM [^{14}C]*N*-acetylglucosamine in the presence of an excess (1 mM) of unlabelled glucose (Glc), fructose (Fru), Xylose (Xyl), glucosamine (GlcN), mannose (Man), sucrose (Scr), galactose (Gal) or GlcNAc (GlcNAc). The activity is expressed relative to the activity detected in the absence of additional unlabelled carbon [332 ± 10 pmol GlcNAc min^{-1} (mg dry weight)$^{-1}$] and was set to 100%. Similar results were obtained in three independent experiments. Standard deviations are displayed by error bars.

In contrast, BAP4 and the parental strain M145 grew normally under all growth conditions. Introduction of pFT185, which harbours a complete *nagE2* gene, led to growth restoration on *N*-acetylglucosamine in mutants BAP5 and BAP6. This suggests that only *nagE2* is needed for growth on GlcNAc.

Transport assays were performed with 20 µM [^{14}C]*N*-acetylglucosamine to establish if *N*-acetylglucosamine transport was affected in the absence of *nagE2*. Expectedly, BAP5 and BAP6 had completely lost uptake of GlcNAc (< 0.010 nmol *N*-acetylglucosamine per minute per mg dry weight; Fig. 2A). In contrast, the *nagE1* mutant BAP4 and its congenic wild-type partner *S. coelicolor* M145 showed inducible transport characteristics, with approximately fourfold induction in the presence of *N*-acetylglucosamine (0.094 ± 0.008 and 0.355 ± 0.017 nmol *N*-acetylglucosamine per minute per mg dry weight), in the absence and presence of *N*-acetylglucosamine, respectively, for *S. coelicolor* wild-type M145. To see whether *S. coelicolor* may also possess a low-affinity uptake system for *N*-acetylglucosamine, BAP5 was examined in transport assays at concentrations up to 2 mM, but this failed to reveal detectable transport activity. Furthermore, uptake rates of the wild type at concentrations from 0.5 mM to 2 mM were comparable to those obtained at lower concentrations. Apparently, the immediate response to *N*-acetylglucosamine is mediated through the PTS permease NagE2.

Induction of the genes of the *N*-acetylglucosamine regulon (*nag*) should therefore be lost in *nagE2* mutants. This was examined by measuring the amount of the cytoplasmic PTS component HPr in mycelia grown on glycerol, GlcNAc or the GlcNAc polymer chitin. Figure 2B shows that GlcNAc-dependent induction of HPr expression observed in *S. coelicolor* M145 was lost in *nagE2* null strains (BAP5 and BAP6), while it was unaffected in the *nagE1* null mutant (BAP4). In all cases, no significant expression of HPr was observed with glycerol or chitin as the sole carbon source.

Table 1. PTS-dependent *N*-acetylglucosamine phosphorylation.

Soluble fraction		Membrane fraction		
Strain	GlcNAc present during growth	Strain	GlcNAc present during growth	Phosphorylation activity [nmol GlcNAc-P (mg membranes)$^{-1}$ min^{-1}]
M145	+	M145	–	32 ± 1.6
M145	+	M145	+	260 ± 8.4
M145	+	BAP4	–	45 ± 2.0
M145	+	BAP4	+	245 ± 7.2
M145	+	BAP5	+	< 10
M145	+	BAP6	+	< 10
BAP5	–	M145	–	40 ± 2.6
BAP5	–	M145	+	36 ± 1.8
BAP5	+	M145	+	43 ± 1.5

Cells of M145, BAP4 (Δ*nagE1*), BAP5 (Δ*nagE2*) and BAP6 (Δ*nagE1* Δ*nagE2*) were grown in mineral medium supplemented with glycerol (–) or glycerol plus GlcNAc (+). Cytoplasmic protein fractions were applied at 250 μg, while membrane vesicles-containing preparations were at 30 μg of protein per assay volume of 0.1 ml. PEP-dependent phosphorylation assays with solely cytoplasmic protein fractions or membrane vesicles-containing preparations exhibited no activity (data not shown).

NagE2 is a high-affinity PTS permease with narrow substrate specificity

Since NagE2 is the only transporter for *N*-acetylglucosamine in *S. coelicolor* – at least under the conditions used – we could monitor the kinetics of transport and substrate specificity in wild-type mycelia grown on *N*-acetylglucosamine. Analysis of transport rates at various substrate concentrations revealed a K_m value of 2.6 μM and a V_{max} of 0.55 nmol min^{-1} (mg dry weight)$^{-1}$, which is indicative for a high-affinity uptake system (Fig. 2C). When mycelia were incubated with an excess of diverse unlabelled sugars (50-fold excess, 1 mM) (Fig. 2D), we found that only addition of *N*-acetylglucosamine led to a significant substrate competition with radiolabelled *N*-acetylglucosamine. The experimental set-up revealed a reduction of the transport rate by 96%. Hence, NagE2 exhibits a narrow specificity for the carbon and nitrogen source *N*-acetylglucosamine.

Reconstitution of the complete N*-acetylglucosamine PTS permease complex*

To characterize the phosphotransfer reactions in the *N*-acetylglucosamine PTS permease complex, which consists of four phosphotransferase domains (EI, HPr, IIA, IIB) and one transmembrane protein (IIC), we reconstituted the interactions from components that included purified protein. Extracts of cytoplasmic proteins from mycelia grown in the presence and absence of *N*-acetylglucosamine were combined with limiting amounts of IIC^{GlcNAc}-containing membranes (Table 1). While addition of the membrane fraction from *nagE1* mutant BAP4 to soluble proteins of wild-type M145 cells affected normal PTS-dependent phosphorylation of *N*-acetylglucosamine, such phosphorylation was not observed when membranes of the *nagE2* mutant BAP5 or of the *nagE1/E2* double mutant BAP6 were mixed with soluble proteins of the wild type. The activity was 5.4-fold enhanced when BAP4 cells were challenged with *N*-acetylglucosamine. Soluble protein fractions of BAP5 cells grown in the presence of *N*-acetylglucosamine or glycerol showed basal phosphorylation activity when incubated with NagE2-containing membranes. This indicated that the two mutants expressed basal amounts of EI, HPr and IIA^{Crr}, which were no longer inducible by *N*-acetylglucosamine due to the lack of the *N*-acetylglucosamine IIC^{GlcNAc} domain. Combination of cytoplasmic proteins and membranes from wild-type cells showed again GlcNAc-dependent phosphorylation that correlated with the transport depicted in Fig. 2A.

Nevertheless, an enzyme IIB^{GlcNAc} was still missing in the PTS phosphotransfer chain. The most likely candidate was the gene product of *nagF* [SCO2905; originally designated *malX2* (Bertram *et al*., 2004)] located two genes upstream of *nagE2* (Fig. 1). Recombinant NagF was overproduced in *E. coli* M15 (pREP4, pFT53 *nagF*$^+$) and purified by immobilized metal affinity chromatography. PEP-dependent phosphorylation of NagF required the phosphotransferases EI, HPr and IIA^{Crr}, as demonstrated by its faster migration in a native polyacrylamide gel as a result of the additional negative charge provided by the phosphoryl group (compare lanes 2, 5 and 6).

To unequivocally ascertain that we had all the components required to constitute the GlcNAc transporter, the entire PTS permease complex was reconstituted *in vitro* by the addition of single-protein components (Fig. 3B). PEP-dependent phosphorylation of [^{14}C]*N*-acetylglucosamine occurred in the presence of EI, HPr, IIA^{Crr}, NagF combined with NagE2-containing membrane vesicles, while no phosphorylation occurred when NagF was omitted. This shows that NagF is indeed the IIB^{GlcNAc} domain of the GlcNAc PTS permease.

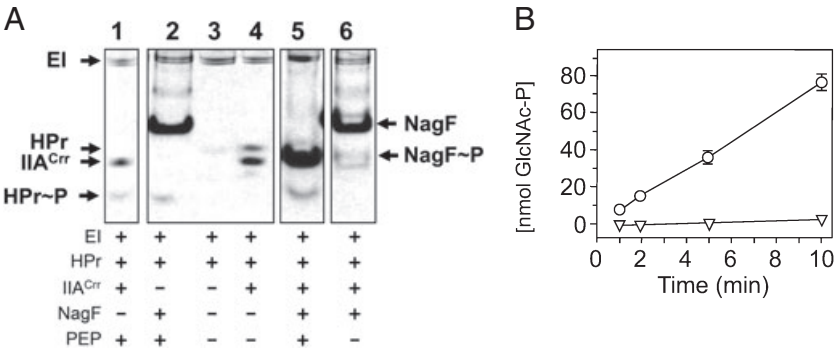

Fig. 3. A. *In vitro* phosphorylation of IIB^{GlcNAc} (NagF). The 15% native polyacrylamide gel shows the Coomassie brilliant blue-stained proteins EI, HPr, IIA^{Crr} and NagF. PEP-dependent phosphorylation was carried out with 20 pmol of histidine-tagged EI, 50 pmol of histidine-tagged HPr, 30 pmol of histidine-tagged IIA^{Crr} and 500 pmol of histidine-tagged NagF in the absence or presence of 1 mM PEP. The combinations of the proteins are indicated below each lane. EI, HPr, IIA^{Crr} and NagF and their phosphorylated forms are assigned by arrows.
B. Reconstitution of the GlcNAc-specific PTS permease complex. Enzyme IIB^{GlcNAc} activity was monitored by PEP-dependent GlcNAc phosphorylation. Membranes containing IIC^{GlcNAc} (30 μg), histidine-tagged EI (50 pmol), histidine-tagged HPr (50 pmol) and histidine-tagged IIA^{Crr} (50 pmol) were incubated in the presence of 100 pmol of histidine-tagged IIB^{GlcNAc} (NagF) (O) or in its absence (∇). Standard deviations given by error bars indicate the mean of triplicate measurements from two independently conducted experiments.

nagF *and* nagE2 *are induced by* N-*acetylglucosamine*

The experiments described above suggest that expression of *nagF* and *nagE2* is triggered by *N*-acetyglucosamine. To analyse this at the level of gene transcription, we performed semi-quantitative RT-PCR experiments of the *nagF-nagE1-nagE2* locus. RNA from mycelia grown in the presence of glycerol revealed signals for *nagF*, *nagE1* and *nagE2* mRNAs. The *nagF* and *nagE2* transcript levels were enhanced in RNA samples obtained from cells grown in the presence of GlcNAc, while *nagE1* transcription was not induced by GlcNAc (Fig. 4). Consequently, these data support our findings that only *nagE2* (and not *nagE1*) encodes a PTS-specific enzyme IIC^{GlcNAc}.

NagE2 is essential for C-signalling between vegetative growth and development

We recently showed that *S. coelicolor* is blocked in development and antibiotic production when grown on solid media in the presences of GlcNAc (Rigali *et al.*, 2006; 2008). Interestingly, there is a sharp transition for the developmental effect of GlcNAc with development and antibiotic production at lower concentrations, and vegetative arrest and almost complete block of antibiotic production above 10 mM.

A bioassay was applied to test the ability of BAP4, BAP5 and BAP6 to sustain GlcNAc signalling. Plating *S. coelicolor* M145 and its mutant derivatives BAP4 (Δ*nagE1*), BAP5 (Δ*nagE2*) and BAP6 (Δ*nagE1 nagE2*) on R2YE agar plates supplemented with increasing amounts of GlcNAc revealed that BAP5 and BAP6, neither of which can produce the NagE2 component, had become insensitive to GlcNAc-mediated signalling; that is to say, both strains sporulated normally (Fig. 5A).

To establish if NagF is indeed an essential component of the GlcNAc transporter complex, and that its function cannot be taken over by other proteins in *S. coelicolor*, we created a *nagF* gene replacement mutant, BAP31, using the same strategy as for BAP4-6. Expectedly, like *nagE2* mutants, *nagF* mutants had become insensitive to *N*-acetylglucosamine (Fig. 5B), underlining that transport through the PTS permease is crucial for C–signalling of N-acetylglucosamine. Thus, transport through the functional PTS permease is required for C-signalling of *N*-acetylglucosamine.

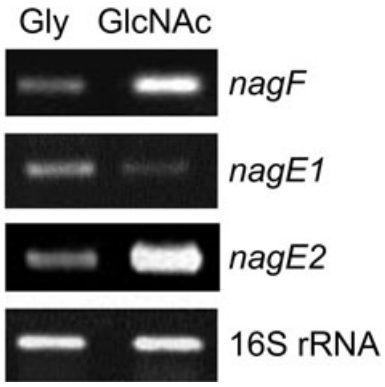

Fig. 4. Transcriptional analysis of the *nagF-nagE1-nagE2* locus. Total RNA was prepared from cultures grown on mineral medium supplemented with 50 mM glycerol (Gly) or glycerol plus GlcNAc (GlcNAc). A 1% agarose gel shows RT-PCR amplification products after the 30th cycle of the PCR reaction with oligonucleotides specific for the indicated transcripts. The enhanced expression levels of *nagF* and *nagE2* indicate their GlcNAc-dependent regulation.

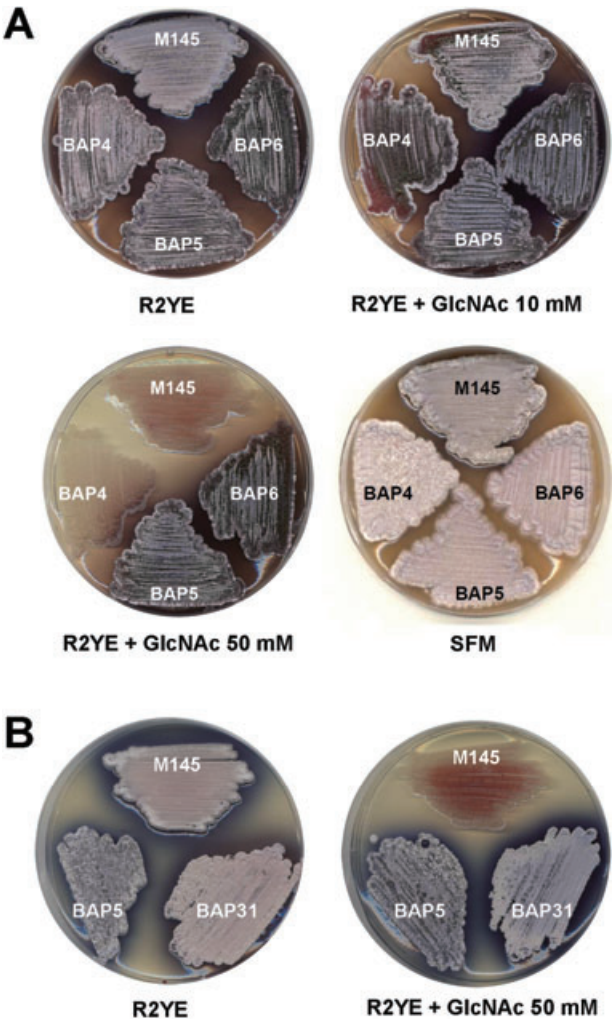

Fig. 5. *nagE2* and *nagF* mutants are insensitive to GlcNAc.
A. *S. coelicolor* M145 and its *nagE1* mutant (BAP4), *nagE2* mutant (BAP5) and *nagE1nagE2* double mutant (BAP6) were grown on SFM agar plates or on R2YE agar plates with increasing concentrations of GlcNAc (GlcNAc; 0, 1, 5, 10, 20 or 50 mM). On SFM agar and on R2YE with up to 10 mM GlcNAc, all strains developed normally. At 50 mM GlcNAc, M145 and BAP4 were locked in the vegetative state, while under the same conditions mutants lacking the GlcNAc transporter NagE2 (BAP5 and BAP6) develop normally.
B. This figure shows a similar experiment as in (A), but now with M145 and its *nagE2* and *nagF* mutants. The sporulating phenotype of the *nagF* mutant on higher concentrations of *N*-acetylglucosamine underlines that NagF is as crucial for GlcNAc signalling as NagE2.

nagE2 *is transcriptionally activated by AtrA*

We reported in a recent publication that DasR represses all studied *nag* and *pts* genes (Rigali *et al.*, 2006). Interestingly, besides two adjacent DasR-responsive elements (*dre*) that form an inverted repeat (nt positions –49/–32 relative to the *nagE2* translational start), we identified a sequence (GGAATCACGGGTTCC at –88/–73 relative to the start of *nagE2*) that showed similarity to a *cis*-acting regulatory element upstream of *actII*-ORF4 (GGAATGC CAGATTCT), the gene for the pathway-specific activator of the genes for the blue-pigmented antibiotic actinorhodin (Fig. 1). In addition to the sequence similarity, both sequences contain an imperfect inverted repeat. The *cis*-element upstream of *actII*-ORF4 is a known target for the TetR-family transcriptional activator AtrA, which switches on actinorhodin production via *trans*-activation of *actII*-ORF4 (Uguru *et al.*, 2005). To analyse whether the putative *cis*-acting element upstream of *nagE2* is indeed bound by AtrA, we performed electrophoretic mobility shift assays (EMSAs) using purified recombinant AtrA (Uguru *et al.*, 2005). A probe encompassing the *nagE2* promoter region was bound efficiently by purified AtrA, with around 50% of the DNA bound when 60 nM AtrA was used (Fig. 6A). As a negative control we used a DNA fragment corresponding to the +56/+333 region of the *malR* gene

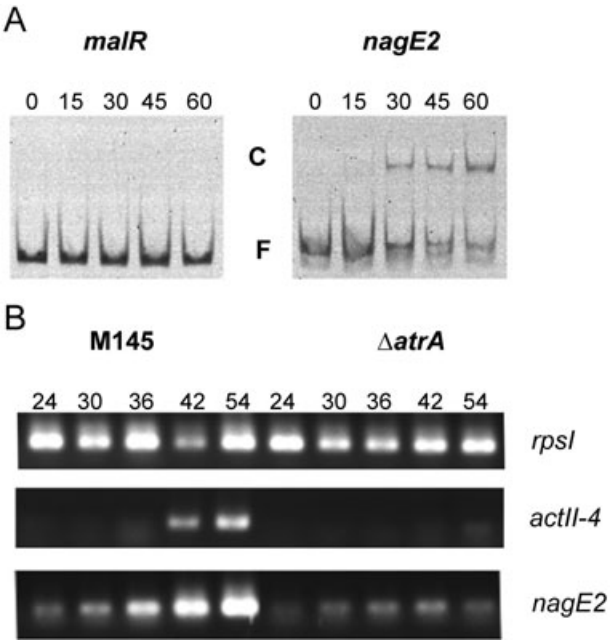

Fig. 6. AtrA transactivates *nagE2* transcription.
A. EMSAs showing binding of AtrA to the *nagE2* promoter. DNA probes encompassing the AtrA consensus-like sequence in the *nagE2* promoter region and an internal fragment of *malR* (negative control) were incubated with increasing amounts of purified His-tagged AtrA (0, 15, 30, 45 or 60 nM). C: AtrA/DNA complex; F: free DNA.
B. Transcription of the pathway-specific activator gene for actinorhodin biosynthesis (*actII*-ORF4; middle row) and gene encoding the PTS component IIC^{GlcNAc} (*nagE2*; bottom row) was analysed by semi-quantitative RT-PCR. Samples were collected from *S. coelicolor* M145 and the *atrA* mutant grown on MM mannitol agar plates after 24 h, 30 h (vegetative growth), 36 h (initiation of aerial growth), 42 h (aerial growth) and 54 h (aerial growth and spores). *rpsI* (encoding ribosomal protein S9; top row) was used as the control for RNA integrity. Reactions without reverse transcriptase were carried out on all RNA samples to establish the lack of contaminating DNA (not shown). Note that the mRNAs of *actII*-ORF4 and *nagE2* are strongly reduced in the *atrA* mutant, indicating that their transcription depends on AtrA.

that had previously been shown not to be bound by AtrA, at least to a detectable level (van Wezel *et al*., 1997a,b). Transcriptional analysis of RNA isolated from mycelia grown on minimal medium agar plates with mannitol as the sole carbon source revealed that transcription of *nagE2* is strongly reduced in an *atrA* mutant at all time points (vegetative growth, aerial growth and sporulation; Fig. 6B).

In contrast, the transcription of ribosomal S11 protein-encoding *rpsI*, which was included as a control, was constitutive and independent of *atrA*. We also analysed transcription of *actII*-ORF4, the cluster-situated regulator of the actinorhodin genes, under these conditions. As reported previously for mycelium grown on R5 plates, the transcription of *actII*-ORF4 was induced during development in the wild-type cells and reduced in the *atrA* mutant (Uguru *et al*., 2005). Indeed, under the conditions used here, the *actII*-ORF4 transcript could not be detected in the *atrA* mutant (Fig. 6B). These experiments established that AtrA *trans*-activates both *actII*-ORF4 and *nagE2*, while we previously showed that both these genes are repressed by DasR under the same conditions (Rigali *et al*., 2008). This immediately suggests that the regulatory networks of AtrA and DasR, which have otherwise little overlap (our unpublished data), have opposing effects on actinorhodin production.

Discussion

N-acetylglucosamine and related aminosugars are important carbon and nitrogen sources for streptomycetes, especially because the polymer chitin serves as an abundant substrate for these organisms in the natural environment of the soil. In this communication, we provide a molecular dissection of GlcNAc transport of the model organism *S. coelicolor* A3(2). We found that this bacterium has one major uptake system for this nutrient, the IIB^{GlcNAc}/IIC^{GlcNAc} PTS permease encoded by *nagF* and *nagE2*. Our observation that this permease is subject to multi-level control by pleiotropic and globally acting transcription factors (namely AtrA and DasR) of antibiotic gene synthesis suggests a tight connection between nutrition and morphogenesis, and shows that the PTS has a broad impact on major decisions in the *Streptomyces* life cycle.

The *nagF-nagE1-nagE2* gene cluster is conserved in streptomycetes, including *Streptomyces avermitilis* and *S. olivaceoviridis* (Wang *et al*., 2002; Ikeda *et al*., 2003). This conservation suggests a common function. The two *nagE* genes most likely arose from gene duplication. While conserved in evolution, despite 65% amino acid sequence identity to NagE2, NagE1 cannot sustain GlcNAc uptake. The *S. olivaceoviridis* NagE2 homologue (PtsC2) was identified as a PTS permease with similar transport affinity and substrate specificity as *S. coelicolor* NagE2 (Wang *et al*., 2002). Interestingly, *S. olivaceoviridis* can also transport GlcNAc through the ATP-dependent ABC porter NgcEFG (Xiao *et al*., 2002). A homologous *ngc* (SCO6005-6007) operon exists in *S. coelicolor*, with between 35% and 54% protein sequence identity for the different components at the protein level (Bertram *et al*., 2004). However, deletion of *nagE2* led to complete loss of uptake, suggesting that in contrast to *S. olivaceoviridis*, the Ngc permease of *S. coelicolor* does not transport GlcNAc.

We show here that the preceding gene *nagF* encodes the structural IIB domain of the permease complex. The reconstitution experiment with all purified PTS proteins demonstrated unequivocally that the PTS permease is composed of the components EI, HPr, IIA^{Crr}, IIB^{GlcNAc} and IIC^{GlcNAc}, encoded by *ptsI*, *ptsH*, *crr*, *nagF* and *nagE2* respectively. The involvement of IIA^{Crr} as the IIA domain of the GlcNAc permease is in this context of special interest. This protein resembles the global-acting enzyme IIA^{Glc} of *E. coli* that (i) operates in PTS-dependent uptake of the carbon sources glucose, sucrose, trehalose, (ii) senses the nutritional state of the cell by responding to the internal ratio of the metabolites pyruvate/phophoenolpyruvate, (iii) triggers the stress response sigma factor RpoS, (iv) interacts with cellular enzymes and uptake systems to control their activities, and (v) participates in global gene regulation by controlling cAMP production and thus the global transcription factor CRP (cAMP receptor protein) (Brückner and Titgemeyer, 2002; Kamionka *et al*., 2002; Saier, 1989). As far as we know now, enzyme IIA^{Crr} indeed has multiple functions in *S. coelicolor*. It phosphorylates a second PTS permease, namely the enzyme $IIBC^{MalX1}$ (our unpublished results), and notably inactivation of IIA^{Crr} causes complete loss of development, strongly suggesting a role that goes way beyond specific sugar transport (Rigali *et al*., 2006). Hence, the complete functional repertoire of the phosphotransferase IIA^{Crr} deserves further attention.

Closer inspection of the previously reported effect of GlcNAc on the development of *S. coelicolor* (Rigali *et al*., 2006) revealed that there is a remarkably sharp transition between the effective and the non-effective concentration of this PTS sugar. At concentrations below 10 mM GlcNAc does not noticeably affect development, at 10 mM sporulation is reduced and at 20 mM or higher the cells are locked in the vegetative state (no aerial hyphae or spores). This effect was lost in *nagE2* mutants, where normal development was obtained even at very high GlcNAc concentrations, which corroborates our observation that *nagE2* is essential for GlcNAc-mediated signalization.

Surprisingly, transcriptional analysis of the *nagF-nagE1-nagE2* gene cluster revealed that GlcNAc induces *nagF* and *nagE2* but not *nagE1*, even though the latter gene lies in the middle of the gene cluster. The regulation

is mediated through the global regulator DasR, as it was shown that DasR binds to *dre* sites (DasR-responsive elements) (Rigali *et al*., 2004) that occur in the upstream regions of *nagF* and *nagE2* as well as in front of the other *pts* genes needed for the permease complex. Furthermore, *dre* elements are part of the promoter sites for numerous genes of the chitinolytic system and are located upstream of several antibiotic regulatory genes (Colson *et al*., 2007; Rigali *et al*., 2006; 2008). In agreement with this, *dasR* knock-out mutants show constitutive uptake of GlcNAc (Rigali *et al*., 2006); additionally, sporulation is lost and control of antibiotic production is strongly disturbed (Rigali *et al*., 2008). Inspection of the corresponding regions of *S. avermitilis* and *S. olivaceoviridis* revealed the presence of conserved *dre* sites in these species, indicating a common way of global control.

The pleiotropic effect of GlcNAc on development and antibiotic production suggests that the decision to import or exclude this sugar is a critical control point in the life cycle of streptomycetes, and it was anticipated that multilevel control of NagE2 would exist. Examination of the *nagE2* promoter region identified a sequence (GGAAT CACGGGTTCC at –88/–73 relative to the start of *nagE2*) that showed striking similarity with *cis*-acting elements bound by the TetR-family regulator AtrA, which activates the production of the blue-pigmented polyketide antibiotic actinorhodin by *trans*-activation of the cluster-situated regulator gene *actII*-ORF4 (Uguru *et al*., 2005). Moreover, we established that AtrA binds to the predicted AtrA binding site upstream of *nagE2* (Fig. 6A) and that *nagE2* transcript levels are strongly reduced in an *atrA* knock-out strain, demonstrating that AtrA *trans*-activates *nagE2* transcription (Fig. 6B). Thus, *atrA* has the potential to be an important regulator of *N*-acetylglucosamine transport and thus affects the production of glucosamine-6-phosphate (GlcN-6-P), which is the inducer of DasR, such that its DNA-binding activity is reduced. Together with the recent finding that DasR, like AtrA, binds to and regulates the promoter of *actII*-ORF4 (Rigali *et al*., 2008), the above results suggest that actinorhodin production and *N*-acetylglucosamine metabolism are intimately co-ordinated. A bioinformatic scan of the *S. coelicolor* genome using the predetector algorithm (Hiard *et al*., 2007) has revealed that, although there are around 250 *dre* sites and 25 AtrA-responsive elements, there is unlikely to be substantial overlap between these regulons beyond *actII-ORF4* and *nagE* (our unpublished results).

In conclusion and as depicted in the model shown in Fig. 7, the GlcNAc-specific PTS of *S. coelicolor* was dissected and biochemically reconstituted, providing conclusive evidence that NagE2 is the high-affinity transporter for GlcNAc. Loss of this nutrient permease relieves the developmental signalling by GlcNAc, indicating that NagE2 is the only transporter for GlcNAc, thus controlling

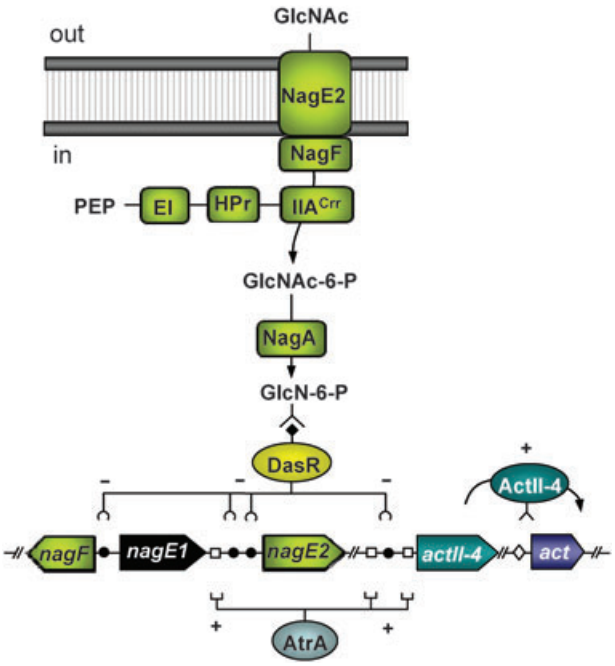

Fig. 7. Gatekeeper NagE2 and caretakers DasR and AtrA. *N*-acetylglucosamine (GlcNAc) enters the cytoplasm and is phosphorylated via the GlcNAc-specific phosphoenolpyruvate (PEP)-dependent PTS permease complex that is composed of general phosphotransferases EI, HPr and IIA^{Crr}, and the specific IIB and IIC components, NagF and NagE2 respectively. *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P) is further deacetylated by NagA (GlcNAc-6-P deacetylase). The resulting glucosamine-6-phosphate (GlcN-6-P) is an allosteric effecter of DasR that inhibits its DNA-binding ability, resulting in loss of transcriptional repression of *nagF*, *nagE2* and *act*II-ORF4 which encodes the pathway-specific transcriptional activators of the actinorhodin (Act) biosynthetic clusters. Beside the negative control exerted by DasR, *nagE2* and *act*II-ORF4 are activated by AtrA. (●), (□) and (◇) are symbols for *cis*-acting elements bound by DasR, AtrA and ActII-4 regulators respectively. The model represents the situation under starvation conditions (such as on MM mannitol plates), where GlcNAc ultimately activates antibiotic production and development. Under rich conditions the opposite is true (Rigali *et al*., 2008).

the first step of the GlcNAc-signalling pathway: GlcNAc transport → GlcNAc-6-P → GlcN-6-P → DasR → onset of antibiotic production. The AtrA and DasR regulons overlap only for the first *(nagE2*) and last (*actII-ORF4*) genes of the signalling cascade. However, by activating GlcNAc transport, AtrA also stimulates the accumulation of GlcN-6-P, an inhibitor of DasR DNA-binding activity, and thus affects post-translational control of *dasR*. In this way, AtrA counteracts the repression by DasR of a multitude of genes, including the *nag* and *pts* regulons, the chitinolytic system, many ABC sugar transporters as well as key developmental and antibiotic biosynthetic genes. This sheds important new light on how streptomycetes sense and respond to changes in the nutritional status of their habitat and relay this information to control the onset of development and antibiotic production.

Table 2. Bacterial strains and plasmids.

Strain or plasmid	Relevant genotype and phenotype	Reference
Bacterial strains		
Escherichia coli		
DH5a	Recipient for cloning experiments	Sambrook *et al.* (1989)
ET12567	Produces non-methylated DNA; Cam^R, Tet^R	Kieser *et al.* (2000)
M15 (pREP4)	Host for overexpression	Galinier *et al.* (1997)
Streptomyces coelicolor		
M145	$SCP1^-$, $SCP2^-$, prototroph	Kieser *et al.* (2000)
BAP2	M145 *crr::aacC4*, Apr^R	Nothaft *et al.* (2003a)
BAP3	M145 *ptsI::aacC4*, Apr^R	Nothaft *et al.* (2003a)
BAP4	M145 *nagE1::aacC4*, Apr^R	This study
BAP5	M145 *nagE2::aacC4* Apr^R	This study
BAP6	M145 *nagE1::aacC4::nagE2*, Apr^R	This study
BAP31	M145 *nagF::aacC4* Apr^R	This study
Plasmids		
pBluescriptSK+	Cloning vector, ColE1 replicon, Amp^R	Stratagene
pHLW1	Plasmid containing *aacC4* resistance gene, Apr^R, Amp^R	Udo Wehmeier
pWHM3	Cloning vector, ColE1 replicon, pSG5 replicon, Amp^R, Tsr^R	Vara *et al.* (1989b)
pQE30/31	T5-cloning vector, Col E1 replicon, expression of N-terminally his_6-tagged proteins, Amp^R	Qiagen
pFT3	Overexpression vector for *ptsH* of *S. coelicolor*, Amp^R	Parche *et al.* (1999)
pFT19	pBlueskriptSK(+) with 277 bp *malX1* B-domain fragment, Amp^R	This study
pFT20	pBlueskriptSK(+) with 250 bp *nagF* fragment, Amp^R	This study
pFT35	Overexpresssion vector for *ptsI* of *S. coelicolor*, Amp^R	Nothaft *et al.* (2003a)
pFT41	Overexpresssion vector for *crr* of *S. coelicolor*, Amp^R	Kamionka *et al.* (2002)
pFT53	pQE31 with 308 bp BamHI–HindIII *malX1* fragment, Amp^R	This study
pFT54	pQE 31 with 308 bp KpnI–PstI *nagF* fragment, Amp^R	This study
pFT113	3 kb HincII *nagE1-nagE2* region from SCE19A cloned into EcoRV site of pBlueskriptSK(+), Amp^R	This study
pFT114	*aacC4* gene from pHLW1 cut with BamHI, treated with T4-Pol., cloned into AccIII, AatII sites of pFT113 treated with T4 Pol., Amp^R, Apr^R	This study
pFT115	*aacC4* gene from pHLW1 cut with BamHI, treated with T4-Pol., cloned into AccIII site of pFT113, treated with T4 Pol., Amp^R, Apr^R	This study
pFT116	*aacC4* gene from pHLW1 cut with BamHI, treated with T4-Pol., cloned into StuI–SphI sites of pFT113, treated with T4 Pol., Amp^R, Apr^R	This study
pFT117	pWHM3 with HindIII–EcoRI (*nagE1::aacC4::nagE2*) fragment from pFT114, Amp^R, Apr^R, Tsr^R	This study
pFT118	pWHM3 with HindIII–EcoRI (*nagE1::aacC4*) fragment from pFT114, Amp^R, Apr^R, Tsr^R	This study
pFT119	pWHM3 with HindIII–EcoRI (*nagE2::aacC4*) fragment from pFT114, Amp^R, Apr^R, Tsr^R	This study

Amp: ampicillin, used at 100 mg l^{-1}; Tet: tetracycline, used at 12 mg l^{-1}; Tsr: thiostrepton, used at 25 mg l^{-1}; Apr: apramycine, used at 25 mg l^{-1}; Cam: chloramphenicol, used at 25 mg l^{-1}.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table 2. Cells of *S. coelicolor* were grown for 30–72 h under static conditions or vigorous shaking at 28°C using tryptic soy broth without dextrose as complex medium (TSB, Difco) or mineral medium (Nothaft *et al.*, 2003b). Carbohydrates and casamino acids (CAA) were added to final concentrations of 50 mM and 0.1%. Mineral medium agar plates were prepared with only 0.6% Noble agar (Difco) to reduce growth on agar as a carbon source. Morphological characterization was inferred from colony morphology of strains grown on SFM (soya flour mannitol) and R2YE agar plates (Kieser *et al.*, 2000), and on six-well tissue culture plates (Sarstedt) with R2YE agar supplemented with 0–25 mM *N*-acetylglucosamine. Cells of *E. coli* were grown in Luria–Bertani broth (LB) at 37°C.

Construction of mutants

Cosmid SCE19A (kindly provided by Matthias Redenbach) was digested with HincII and a 2.984 bp fragment containing the complete *nagE1-nagE2* gene locus was ligated into the EcoRV site of plasmid pBlueskriptSK(+). The resulting plasmid, pFT113, was digested in three parallel experiments to remove sections of *nagE1*, *nagE2* or both. The plasmid was digested either (i) with AccIII to delete nt positions 348–360 of the 1296 bp *nagE1*, or (ii) with StuI and SphI to remove nt positions 335–677 of the 1251 bp *nagE2*, or (iii) with AccIII and AatII to remove the section from nt position 348 of *nagE1* to nt position 742 of *nagE2* respectively. The three linearized plasmid derivatives were then blunt-ended with T4 DNA polymerase. A 1.3 kb fragment containing the *aacC4* cassette for apramycin resistance obtained from pHLW1 (kindly provided by Udo Wehmeier) was inserted as a blunt-ended BamHI fragment. Plasmids containing the *aacC4* gene

transcribed in opposite direction to *nagE1* and *nagE2* were termed pFT115, pFT116 and pFT114 respectively. Plasmids pFT118 (*nagEI::aacC4*), pFT119 (*nagE2::aacC4*) and pFT117 (*nagE1′::aacC4::′nagE2*) were created by isolation of the mutated *nagE1-nagE2* alleles from HindIII–EcoRI-digested pFT115, pFT116 and pFT114 and subsequent fragment insertion into vector pWHM3 (Vara *et al.*, 1989a) digested with the same enzymes. pFT118, pFT119 and pFT117 were used to transform protoplasts of M145 as described (Nothaft *et al.*, 2003b). Four Tsr^S, Apr^R transformant colonies of each chromosomal integration were verified by PCR on genomic DNA of *S. coelicolor* M145 isolated following a method described previously (Kieser *et al.*, 2000), with *aacC4*-specific oligonucleotides HN4 and HN5 and the oligos nagEmut1, nagEmut2 and nagEmut3 (Table S1). Amplification products of 1.4 kb (HN4-nagEmut1) and 1.3 kb (nagEmut2-nagEmut3) for *nagE1::aacC4*, of 2.8 kb (HN4-nagEmut1) and 1.6 kb (HN5-nagEmut2) for *nagE2:aacC4* and of 1.4 kb (HN4-nagEmut1) and 1.2 kb (HN5-nagEmut2) for the double mutant confirmed the correctness of the desired recombinants. The strains were termed BAP4 (*nagE1::aacC4*), BAP5 (*nagE2::aacC4*) and BAP6 (*nagE1′::accC::′nagEII*) respectively. For complementation of *nagE2* mutants, a PCR fragment of 1575 nt with the complete *nagE2* gene and 268 nucleotides of the promoter (upstream) region was generated using oligonucleotides nagE2reg1 and nagE2rev. The fragment was cloned into expression shuttle vector pUWL-KS into the KpnI and XbaI sites, giving pFT185 ($nagE2^+$).

Using essentially the same strategy as for BAP4, BAP5 and BAP6, we constructed a knock-out plasmid for the gene replacement of *nagF* (SCO2905). The −1389/+6 and +232/+1617 regions of *nagF* (nt positions refer to the start of *nagF*) were amplified by PCR, using primer pairs 2905LF-138 and 2905LR+6 and 2905RF+232 and 2905RR+1617 respectively. After cloning into pWHM3 the apramycin resistance cassette *aacC4* was positioned in the middle using engineered XbaI sites. The gene disruption and mutant selection and screening procedures were performed as described above. The *nagF* deletion mutant was termed BAP31.

Overexpression and purification of recombinant PTS proteins

EI, HPr and IIA^{Crr} were prepared as oligohistidine-tagged fusion proteins as described previously (Kamionka *et al.*, 2002; Nothaft *et al.*, 2003a; Parche *et al.*, 1999). For overexpression of NagF as His_6-tag fusion protein, a DNA fragment of 251 bp was amplified by PCR containing the 231 bp *nagF* coding region with 10 bp on either side. Oligonucleotides nagFBD1 and nagFBD2 (Table S1) were used for amplification of *nagF*. The PCR product was ligated into the EcoRV site of plasmid pBlueskriptSK(+) resulting in pFT20. The orientation of the inserted fragment was determined by DNA sequencing, which also revealed sequence identity to the reported *nagF* DNA sequence. pFT54 ($nagF^+$) was obtained by cloning a KpnI–PstI fragment containing *nagF* from pFT20 into pQE31. NagF of *S. coelicolor* A3(2) was overproduced in *E. coli* M15(pREP4) and purification was performed as described previously (Parche *et al.*, 1999). Protein concentrations were determined with the Bio-Rad protein assay. Proteins were stored at −20°C in standard buffer containing 10% glycerol.

RNA preparation and RT-PCR

Total RNA was prepared from mycelium grown in liquid mineral medium in the presence of 50 mM glycerol, fructose, glucose or GlcNAc as described (Nothaft *et al.*, 2003b). For isolation of total RNA from mycelium grown on solid media modified version of the Kirby protocol was used (Noens *et al.*, 2005). The One-step RT-PCR Kit (Qiagen) was used and master mixes were prepared following the manufacturer's instructions. Oligonucleotides for RT-PCR experiments (Table S1) were nagFBD1 and nagFBD2 amplifying the −10/+240 section of *nagF*; RTnagEI-1 and RTnagEI-2 amplifying region +523/+1015 of *nagE1*; RTnagEII-1 and RTnagEII-2 amplifying region +267/+755 of *nagE2*; actII-4fli and actII-4rli amplifying region +185/+517 of *actII*-ORF4; 16SrRNA1 and 16SrRNA2 amplifying region +787/+1313 of *rrnA*; and rpsI-fli and rpsI-rli amplifying region +9/+299 of *rpsI*. PCR reactions were carried out for 21, 24, 27 or 30 cycles to allow optimal quantification of the products which were separated on a 1.2% agarose gel in TAE buffer. RT-PCR experiments without prior reverse transcription were performed to exclude DNA contamination (van Wezel *et al.*, 2005). RT-PCR of 16S rRNA or *rpsI* served as the invariant standards.

Electrophoretic mobility gel shift assay (EMSA)

DNA fragments used for EMSAs were amplified by PCR from the *S. coelicolor* M145 genome. The upstream-located oligonucleotides (Table S1) carried a 5′ FAM label, resulting in fluorescently labelled probes. The 278 bp *malR* probe (+56/+333 relative to the start of the gene) was amplified using malRp1 and malRp2, and the 175 bp *nagE2* probe (−212/−38 relative to the start of the gene) was amplified using nagE2p1 and nagEp2. The *actII*-ORF4 promoter previously shown to be a target of AtrA binding (Uguru *et al.*, 2005) was used as the control (not shown). Binding reactions were performed in TGEK buffer [10 mM Tris-HCl pH 7.9, 10% (v/v) glycerol, 0.1 mM EDTA, 50 mM KCl]. FAM-labelled probes were used at 10 nM concentration, protein concentrations were varied in 15 nM increments from 0 to 60 nM. Electrophoresis was carried out on 20 × 20 cm 6% polyacrylamide gels run overnight at 60 V. EMSA gels were scanned using a Fujifilm Fla-5100 scanner.

Enzyme assays and sugar transport

PEP-dependent phosphorylation of [^{14}C]GlcNAc was assayed with dialysed cell extracts. Enzyme II^{GlcNAc} activity was determined by combining 10 µg of II^{GlcNAc}-containing membrane vesicles with an excess (250 µg) of dialysed membrane-free cell extract as a source for HPr, EI, IIA^{Crr} and IIB. IIB activity was measured by combining 50 pmol of each purified EI, HPr, IIA^{Crr} and 100 pmol of NagF protein with 30 µg of II^{GlcNAc}-containing membrane vesicles. Membrane-free cell extracts were obtained by ultracentrifugation of crude cell extract for 1 h at 110 000 *g* at 4°C. Preparation of membrane vesicles and assay conditions were performed as

described (Parche *et al*., 1999; Nothaft *et al*., 2003b). Protein phosphorylation was carried out in a total volume of 25 µl in a buffer containing 50 mM Tris/HCl (pH 7.5), 15 mM $MgCl_2$ and 300 pmol of PEP for 15 min at 30°C. Routinely, 500 pmol of purified NagF was incubated with 10 pmol of purified EI, 50 pmol of HPr and 40 pmol of IIA^{Crr} respectively. An aliquot of the reaction was subjected to native polyacrylamide gel electrophoresis and proteins were visualized by Coomassie brilliant blue (CBB) staining. No activity or protein phosphorylation was detected in the absence of PEP and/or purified proteins and membrane fractions, indicating the specificity of the assays. Transport of [^{14}C]*N*-acetylglucosamine (6.2 mCi mmol^{-1}) at a final concentration of 20 µM was performed as described (Nothaft *et al*., 2003b). The initial velocity of [^{14}C]*N*-acetylglucosamine incorporation was determined by using GlcNAc at different concentrations (0.5–200 µM; 20 to 2 mCi mmol^{-1}) by withdrawing 1 ml of samples after 1 min of incubation. Inhibition of [^{14}C]*N*-acetylglucosamine transport was carried out with GlcNAc-grown cells in the presence of 1 mM fructose, xylose, glucosamine, mannose, sucrose, galactose or GlcNAc.

Computer analyses

DNA and protein databank searches were performed using the BLAST server of the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov) and the *S. coelicolor* genome page services (http://strepdb.streptomyces.org.uk/). Alignments were generated with CLUSTALW available at http://www.ebi.ac.uk/clustalw (Thompson *et al*., 1994).

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