

The chitobiose-binding protein, DasA, acts as a link between chitin utilization and morphogenesis in *Streptomyces coelicolor*

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Streptomyces are mycelial soil bacteria that undergo a developmental programme that leads to sporulating aerial hyphae. As soil-dwelling bacteria, streptomycetes rely primarily on natural polymers such as cellulose, xylan and chitin for the colonization of their environmental niche and therefore these polysaccharides may play a critical role in monitoring the global nutritional status of the environment. In this work we analysed the role of DasA, the sugar-binding component of the chitobiose ATP-binding cassette transport system, in informing the cell of environmental conditions, and its role in the onset of development and in ensuring correct sporulation. The chromosomal interruption of *dasA* resulted in a carbon-source-dependent vegetative arrest phenotype, and we identified a second DasR-dependent sugar transporter, in addition to the *N*-acetylglucosamine phosphotransferase system (PTS^{GlcNAc}), that relates primary metabolism to development. Under conditions that allowed sporulation, highly aberrant spores with many prematurely produced germ tubes were observed. While GlcNAc locks streptomycetes in the vegetative state, a high extracellular concentration of the GlcNAc polymer chitin has no effect on development. The striking distinction is due to a difference in the transporters responsible for the import of GlcNAc, which enters via the PTS, and of chitin, which enters as the hydrolytic product chitobiose (GlcNAc₂) through the DasABC transporter. A model explaining the role of these two essentially different transport systems in the control of development is provided.

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INTRODUCTION

Streptomyces are Gram-positive soil bacteria with a complex life cycle that starts with the germination of a single spore, which grows out to form a branched mycelium of vegetative hyphae. In adverse environmental conditions, morphological differentiation is initiated by a complex spatio-temporal programme by which aerial hyphae produce chains of hydrophobic spores. Mutant screens identified two classes of developmental mutants, namely those that are locked in the vegetative state and fail to produce aerial hyphae, the so-called bald (*bld*) mutants,

and those that do produce aerial hyphae but fail to complete sporulation, which are called white (*whi*) mutants, reflecting the lack of grey-pigmented spores (Chater, 1972; Hopwood *et al.*, 1970). Perhaps the most important signal for the onset of development is reduced nutrient availability (Chater & Horinouchi, 2003), and this raises the question as to how these nutritional signals are sensed and transmitted to the cell to control the developmental programme. As soil-dwelling organisms, streptomycetes often use hydrolysis products of the abundant polysaccharides cellulose, xylan, and chitin as food sources. The first two are of plant cell-wall origin, and the third mainly originates from the cell walls of fungi and the cuticles of crustaceans or insects. Their presence is a sign of a rich environmental niche, and as abundant carbon sources they may play a critical role in monitoring the

Abbreviations: ABC, ATP-binding cassette; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

A supplementary figure is available with the online version of this paper.

global nutritional status of the environment. We therefore postulated that their respective sensors/transporters might be at the extremity of transduction pathways that correlate primary metabolism to developmental genes. The hydrolysis of these polysaccharides leads to the direct derivative products cellobiose, xylobiose and chitobiose, which in turn are the best inducers of enzymes involved in their self production (Godden *et al.*, 1989; Miyashita *et al.*, 2000).

Recently, we showed that the addition of *N*-acetylglucosamine (GlcNAc), the monomer unit of chitin, on rich medium (R2YE) was able to lock *Streptomyces coelicolor* in the vegetative growth phase and this molecule was therefore considered as a critical signal to the developmental programme (Rigali *et al.*, 2006). GlcNAc is transported via the sugar phosphotransferase system (PTS) (Nothaft *et al.*, 2003) and the five genes involved (*ptsI* for enzyme EI, *ptsH* for HPr, *crr* for enzyme EIIA, *malX2* for enzyme EIIB^{GlcNAc} and *nagE2* for enzyme EIIC^{GlcNAc}) are transcriptionally repressed by the GntR-type regulator DasR (Rigali *et al.*, 2002, 2004, 2006). Previous studies on developmental mutants of streptomycetes have led to the identification of four genes encoding members of the GntR family: *whiH* (Ryding *et al.*, 1998), *dasR* (Rigali *et al.*, 2006), SCO7168 (Hillerich & Westpheling, 2006) and *devA* (Hoskisson *et al.*, 2006), supporting the idea that members of this family are critical control points for development in these micro-organisms. The computational prediction of the DasR regulon in *S. coelicolor* suggests other DasR-dependent transporters, and we investigated the question if, besides the PTS^{GlcNAc}, other DasR-dependent transporters are also involved in the control of morphological differentiation of streptomycetes and if so, what the transported molecules would be.

In a previous communication we showed that DasR is a pleiotropic regulator that is essential for development of *S. coelicolor* (Rigali *et al.*, 2006). After the PTS^{GlcNAc}, one of the known targets of DasR in *S. coelicolor* is a cluster of three genes that compose an ATP-binding cassette (ABC) sugar transporter, i.e. the DasABC system involved in chitobiose transport (Rigali *et al.*, 2004; Seo *et al.*, 2002). This system consists of *dasA* (SCO5232), encoding the chitobiose-binding protein, located upstream of *dasB* (SCO5233) and *dasC* (SCO5234), encoding the respective permeases. Downstream of *dasC*, SCO5235 (*dasD*) encodes

an intracellular β -*N*-acetylglucosaminidase that could hydrolyse the transported chitobiose into the GlcNAc subunits. While the synteny strongly suggests that the SCO5231–SCO5234 genes of *S. coelicolor* correspond to the *dasRABC* gene cluster of *Streptomyces griseus* (Seo *et al.*, 2002), the overall similarity of the primary sequences is very low, namely 33 % for the solute-binding protein DasA, and 32 % and 43 % for the transmembrane proteins DasB and DasC, respectively, suggesting different transported sugars with therefore potentially different repercussions on morphogenesis. The gene encoding the solute-binding protein of *S. griseus* was previously shown to be involved in the control of morphogenesis, as its inactivation led to a bald phenotype (Seo *et al.*, 2002).

In this work we show that the chitobiose-binding component DasA of *S. coelicolor* is also essential for development, thus uncovering another link between sugar metabolism and the start of the developmental programme. We also show that under conditions that allow sporulation by the mutant, highly aberrant spores are produced with many prematurely produced germ tubes, a sign that the role of DasA extends into the latest stages of sporulation. We also show that although GlcNAc (PTS transport-dependent) locks streptomycetes in the vegetative state, a high extracellular concentration of the GlcNAc-polymer chitin (DasABC transport-dependent) has no effect on streptomycete development. We provide a model explaining the role of these two essentially different transport systems in the control of development.

METHODS

Bacterial strains and culture conditions. All media and routine *Streptomyces* techniques are described in the *Streptomyces* manual (Kieser *et al.*, 2000). Liquid cultures of *S. coelicolor* were grown at 28 °C using tryptic soy broth without 1 % glycerol as complex medium (TSB, Difco) or minimal medium (MM). Soy flour mannitol (SFM) medium was used for making spore suspensions. R2YE agar plates were used for regeneration of protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants. Phenotypic characterization of mutants was done on SFM, R2YE and MM agar plates with various carbon sources as indicated in the text. *S. coelicolor* strains are listed and referenced in Table 1. Others streptomycetes used in this study were *Streptomyces antibioticus* IMRU 3720, *Streptomyces limosus* ATCC 19778, *Streptomyces roseosporus* ATCC

Table 1. Strains of *S. coelicolor* used in this study

S. coelicolor M145 was the parental strain for the various mutants.

<i>S. coelicolor</i> strains	Genotype	Reference
M145	Parental strain, SCP1 [−] SCP2 [−] , prototroph	Kieser <i>et al.</i> (2000)
BAP17, Δ <i>glcP1</i>	M145 <i>glcP1::hyg</i>	van Wezel <i>et al.</i> (2005)
BAP28, Δ <i>chiSR</i>	M145 <i>chiS::aacC4::chiR</i>	This work
BAP29, Δ <i>dasR</i>	M145 <i>dasR::aacC4</i>	Rigali <i>et al.</i> (2006)
SAF3, <i>dasA</i> mutant	M145 <i>dasA::aacC4</i>	This work
M145 Δ <i>glk</i>	M145 <i>glk::aacC4</i>	This work

31568, *Streptomyces acrimycini* DSM 40540 and *Streptomyces cacaoi* KCC-S0352.

Construction of the *dasA*, *glk*, and *chiSR* mutants. The derivatives of cosmids SC7E4 and 6E10 carrying Tn5062 insertions in SCO5232 (*dasA*) and SCO2126 (*glk*) (obtained from Paul Dyson, Swansea, UK), generated using the *in vitro* transposition method of Bishop *et al.* (2004), were introduced into *S. coelicolor* M145 by conjugation from *Escherichia coli* ET12567/pUZ8002. Mutants exhibiting the double-crossover phenotype (apramycin-resistant, kanamycin-sensitive) were confirmed by PCR, and designated SAF3 and SAF5 for *dasA* and *glk*, respectively. Verification of the correct recombination events was performed by PCR and Southern hybridization. Saito *et al.* (2007) reported that transcription of *dasBC* is independent of the transcription of *dasA*, which ensures the absence of a polar effect on the *dasBC* genes. For complementation of the *dasA* mutant we used plasmid pSET151 harbouring the *dasA* gene and its promoter region (nt positions -361/+1278 relative to the start of *dasA*). pSET151 lacks a *Streptomyces* origin of replication, and it can therefore be maintained only by integration into the genome. The selection marker of the plasmid is *tsr* for thiostrepton resistance.

The *chiSR* mutant BAP28 (M145 *chiS*::*aacC4*::*chiR*) was created by replacing nucleotides 566–1142 inside the *chiS* gene by the apramycin resistance gene cassette, using pWHM3. This is a multicopy *E. coli*–*Streptomyces* shuttle plasmid that is readily lost when antibiotic pressure (the plasmid harbours the *tsr* gene for thiostrepton resistance) is not maintained. The desired recombinants carry apramycin resistance, but are sensitive to thiostrepton due to loss of the vector sequences after double crossing over. Verification of the correct recombination events was performed by PCR.

Microscopy. Morphological studies of surface-grown aerial hyphae and spores of *S. coelicolor* M145 and SAF3 by cryo-scanning electron microscopy were performed using a JEOL JSM6700F scanning electron microscope. Transmission electron microscopy for analysis of thin sections of hyphae and spores was performed with a Philips EM410 transmission electron microscope. For stereomicroscopy we used an automated Zeiss Lumar V12 fluorescence microscope at 12–150× magnification, or an automated Leica MZ-16FA with up to 180× magnification and a compound lens for maximal 660× magnification, to establish the presence of aerial hyphae and spores. Phase-contrast microscopy was done with a Zeiss standard 25 microscope with 100–1000× magnification. A 5-Mp CCD camera was used for photography. Images were acquired using the supplier's software and processed by Adobe Photoshop CS2 (Version 9.0).

RT-PCR. RNA was isolated from mycelium of *S. coelicolor* M145 and BAP29. Minimal medium plates containing 0.5% mannitol were inoculated with spores on cellophane discs and samples were collected after 30 h (vegetative growth), 42 h (initiation of aerial growth) and 72 h (aerial growth and spores) incubated at 28 °C. RT-PCR analyses were conducted with the Superscript III One-Step RT-PCR kit (Invitrogen). RT-PCRs without reverse transcription were used as a control for absence of residual DNA. For semiquantitative analysis, samples were taken at four-cycle intervals between cycles 24 and 35 to compare non-saturated PCR product formation. Data were verified in three independent experiments. The oligonucleotides used to assess *dasA* transcription were 5'-CGACATGAAAGTGCAGGTGG-3' and 5'-TCGGGCTTGGTCAGCAAAAGC-3'. *rpsI* was used as control (5'-GAGACCACTCCCAGCAGCCGC-3' and 5'-GTAGCGGTTGTCC-AGCTCGAGCA-3').

Enzyme assays. TSB (25 ml volumes) supplemented with 1% (v/v) glycerol was inoculated with either *S. coelicolor* M145 or SAF3 spore suspensions and cultures were grown to the exponential growth phase. Then 200 mg fresh weight was used to inoculate 25 ml MM

with various carbon sources (1%, w/v). Three samples were collected for all conditions after 48 h and mycelia and supernatants were separated. Mycelium was resuspended in water and disrupted by sonication. The BCA protein assay (Pierce) was used for determining protein concentrations. Chitinase activity measurements were performed as described by Zhang *et al.* (2002) using a colorimetric assay with carboxymethylchitin-Remazol Brilliant Violet 5R (Loewe Biochemica) as substrate. β -N-Acetylglucosaminidase activity measurements were performed at 37 °C as described by Kubota *et al.* (2004) using a colorimetric assay with *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide (Sigma-Aldrich) as substrate. Results are expressed as a percentage of the activity measured for wild-type *S. coelicolor* M145 in induced culture conditions (1% chitin) and are the mean of three to six independent cultures.

RESULTS

dasA null mutants have a highly aberrant phenotype

To analyse the role of *dasA* in *S. coelicolor*, a *dasA* null mutant was generated from the parental strain *S. coelicolor* M145 using a derivative of cosmid SC7E4 carrying the Tn5062 transposon (conferring apramycin resistance) inserted into the *dasA* gene at nucleotide position 5692637 of the *S. coelicolor* chromosome (335 nt downstream of the translational start site of *dasA*). This cosmid derivative, generated using the *in vitro* transposition method of Bishop *et al.* (2004), was introduced into *S. coelicolor* by conjugation from *E. coli*. Mutants exhibiting the double-crossover phenotype (apramycin-resistant, kanamycin-sensitive) were confirmed by PCR, and one was designated SAF3. The resulting mutant has the *egfp* gene under the control of the *dasA* promoter (see below). Interestingly, the *dasA* mutant showed medium-dependent development, only failing to produce aerial hyphae and spores on glucose-containing media (MM mannitol + glucose and R2YE agar plates; Fig. 1). Thus, *dasA* is classified as a novel *bld* mutant of *S. coelicolor*. Many of the *bld* mutants have a conditional phenotype, underlining the importance of sugar metabolism and carbon catabolite control in the control of development (Pope *et al.*, 1996; Nodwell *et al.*, 1999). Interestingly, the developmental deficiency was particularly pronounced in sections with high density, while separate colonies of SAF3 eventually produced aerial hyphae and spores at the colony edges (Fig. 1). This suggests that the developmental block is dependent on a quorum-sensing-like mechanism, and is relieved when colony density is lower.

Closer inspection by cryo-scanning electron microscopy showed a strictly bald phenotype when the *dasA* mutant was grown on R2YE agar plates, while the parent M145 produced normal aerial hyphae and spores (Fig. 2a, b). In contrast, on SFM agar plates the *dasA* mutant did produce aerial hyphae and spores, though sporulation was far less abundant than in the parent M145 (Fig. 2c, d). Excitingly, the *dasA* mutant had highly aberrantly shaped spores, which produced branches at a high frequency (Fig. 2e, f). To our knowledge, such a phenotype has not been established before in any

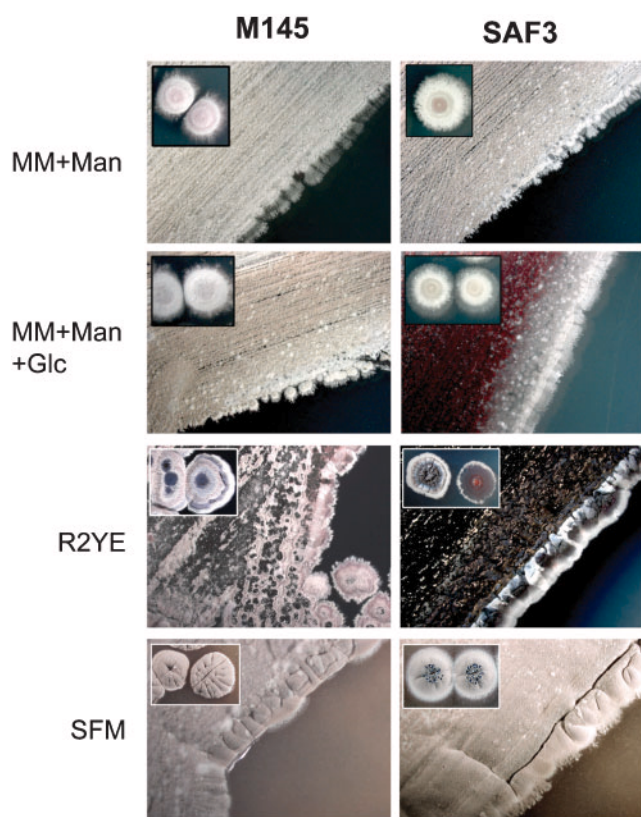


Fig. 1. Stereo light micrographs of M145 and its *dasA* mutant SAF3 on different media. Strains were grown on MM with mannitol or mannitol and glucose, on R2YE and on SFM agar plates for 6 days and photographed using a Zeiss Lumar stereomicroscope. Note how glucose inhibits development inside patches of dense growth, but not around the edges. All photographed sections have a diameter of 3 cm. The insets show representative single colonies (size of the colonies was around 5 mm).

Streptomyces mutant. In contrast, under all conditions the parent M145 showed wild-type sporulation (Fig. 2a, c, e). Closer inspection by transmission electron microscopy again revealed heteromorphic spores that regularly branched (Fig. 3). Hence, the morphological defects were established experimentally by very different microscopic techniques.

Introduction of *dasA* on the integrative plasmid pSET151 into SAF3 reversed the defect (see Supplementary Fig. S1a, available with the online version of this paper), indicating that the inability to form aerial mycelium of the *dasA* mutant was due solely to the disruption of *dasA* and that the procedure used to disrupt *dasA* has no polar effect on *dasBC* expression as previously reported (Saito *et al.*, 2007).

***dasA* expression is repressed by DasR and induced by chitin**

Previously, we showed that the GntR transcriptional regulator DasR was able to bind the DasR responsive

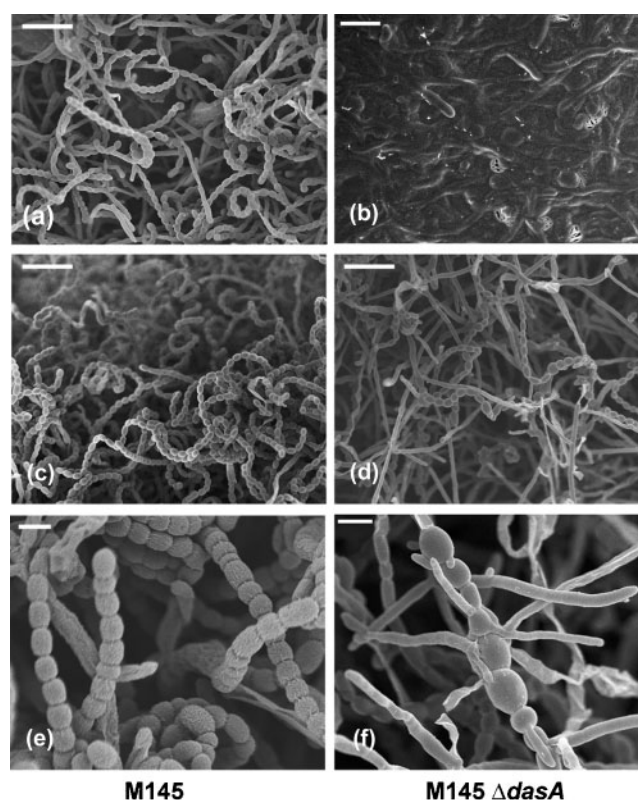


Fig. 2. Scanning electron micrographs of M145 (a, c, e) and its *dasA* mutant SAF3 (b, d, f). The strains were streaked on R2YE (a, b) or SFM (c–f) agar plates, incubated for 5 days and analysed by cryo-scanning electron microscopy. On all media, *S. coelicolor* M145 produced abundant and wild-type spores. In contrast, on R2YE agar plates, the *dasA* mutant SAF3 had a bald phenotype (only vegetative growth), and on SFM agar it produced abundant aerial hyphae and spores, but the spores had many and highly unusual branches protruding from them, most likely reflecting premature germination. Scale bars: (a, c, d), 5 µm; (b), 2 µm; (e, f), 1 µm.

element (*dre*) located 106 nt upstream of the translational start of *dasA* (Colson *et al.*, 2007; Rigali *et al.*, 2004). Semiquantitative RT-PCR on RNA samples of the *dasR* mutant (BAP29) and the parental strain *S. coelicolor* M145 grown on MM + mannitol (0.5%) showed the repressor role of DasR, as *dasA* transcription was increased about twofold throughout the life cycle in BAP29 (Fig. 4).

In order to assess the environmental context that activates *dasA* transcription we took advantage of the fact that the transposon integration resulted in a transcriptional fusion of the promoter of *dasA* (*dasAp*) to the *egfp* gene, so that the activity of *dasAp* can be followed by the expression of EGFP using fluorescence microscopy (Bishop *et al.*, 2004). The *dasA* null mutant (SAF3) was grown on solid MM supplemented with different carbon sources and reporter gene expression was assessed (Fig. 5). The EGFP production was maximal and uniformly distributed within the

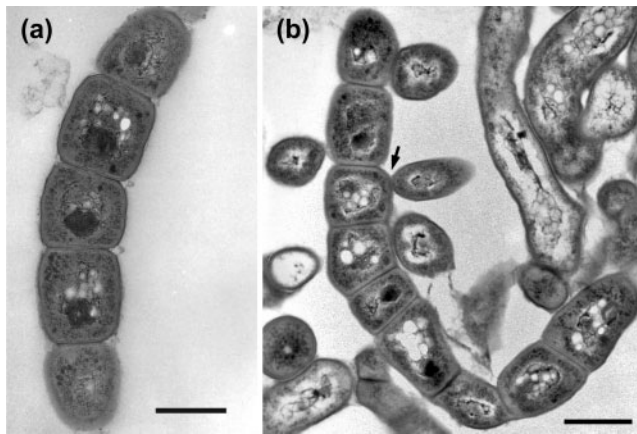


Fig. 3. High-resolution transmission electron micrographs of *dasA* mutant spore chains. Almost mature spore chains of the *dasA* mutant (b) showed several branching sites (example arrowed), most likely due to premature germination of the spores; such branching spores were also visualized by cryo-scanning electron microscopy (see Fig. 2f). Wild-type spores of the parental strain M145 (a) are shown as a control. Scale bars, 1 μ m.

mycelia on MM supplemented with chitin as sole carbon source, while the addition of GlcNAc reduced EGFP expression to the basal level observed on MM + mannitol (Fig. 5). In contrast to what was previously observed (Rigali *et al.*, 2006) and as recently reported by Saito *et al.* (2007), *dasA* is thus far the first known DasR-dependent gene that is not induced but rather repressed by GlcNAc.

To determine the relationship between DasA and chitin utilization, we compared the global chitinolytic activity of SAF3 to that of the parental strain M145 grown on MM agar plates in conditions inducing or repressing chitinase production. As a control, we generated the Δ *chiSR* mutant (BAP28, M145 *chiS::aacC4::chiR*; see Methods) encoding

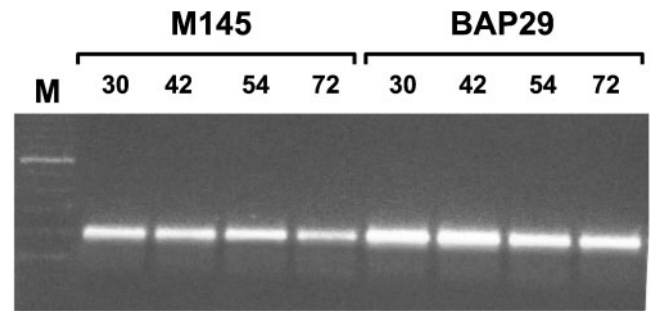


Fig. 4. *dasA* is transcriptionally repressed by DasR. Transcriptional analysis of *dasA* by semiquantitative RT-PCR; RNA was collected after 30, 42, 54 and 72 h growth on solid MM supplemented with mannitol (0.5 %, w/v). 16S rRNA was used as the positive control and reactions without reverse transcriptase as the negative control (not shown). M, size markers.

the two-component system proteins ChiS/ChiR involved in the induction of the chitinolytic system in *S. coelicolor* (Kormanec *et al.*, 2000). Chitinase production was not affected under repressing culture conditions, i.e. in MM + chitin + GlcNAc (1 %) and MM + chitin + glucose (1 %) (Fig. 6). Under inducing culture conditions (MM + chitin), the *dasA* mutant showed halo sizes of chitin hydrolysis about twofold larger than those of the parental strain (Fig. 6). The increased chitinolytic and β -N-acetylglucosaminidase activities were assessed by colorimetric assays (see Methods), and were estimated to be about 30 % higher than those of the wild-type strain after 48 h growth at 28 °C. Complementation of SAF3 with *dasA* cloned into pSET151 restored a normal chitinolytic activity, indicating that overproduction of chitinases in the *dasA* mutant was due solely to the disruption of *dasA* (see Supplementary Fig. S1b). A model which explains the role of DasA in the induction of the chitinolytic system is presented in the Discussion.

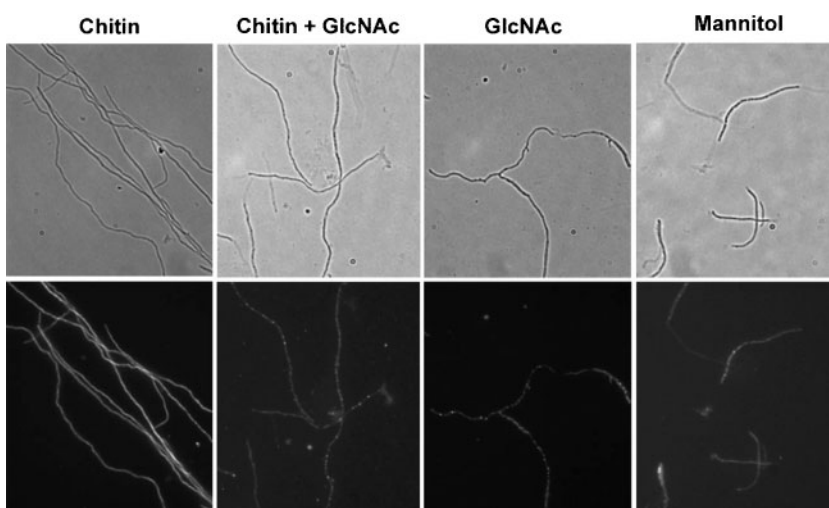


Fig. 5. *dasA* is induced by chitin. Transmission and fluorescence images from confocal microscopy are shown at the top and bottom respectively. The expression of the EGFP protein is under the control of the *dasA* promoter in the SAF3 mutant. EGFP production is maximal and uniformly distributed within the mycelia of SAF3 grown on MM supplemented with chitin (1 %) as sole carbon source. No differences in the levels of fluorescence were observed on chitin + GlcNAc, GlcNAc (1 %) or mannitol (0.5 %).

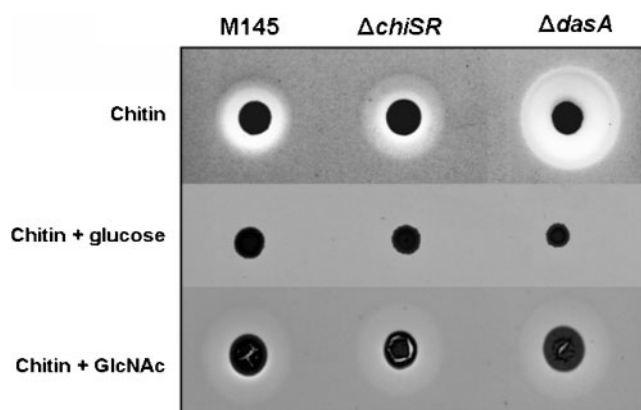


Fig. 6. Global chitinolytic activity of the *dasA* mutant. Spore suspensions of *S. coelicolor* M145 (parental strain), ΔchiSR (BAP28) and ΔdasA (SAF3) were spotted on MM inducing (chitin) or repressing (chitin + GlcNAc or chitin + glucose) the chitinolytic system. The chitinolytic activity is visualized by the haloes around the colonies. All sugars were added to a concentration of 1 % (w/v). M145 and the ΔchiSR mutant were used as positive and negative controls, respectively.

Chitin and the control of development in streptomycetes

The bald phenotype of both *dasR* and *dasA* mutants suggested an association between nutrient utilization –

specifically of monomer and polymer forms of GlcNAc – and development. This correlation was previously demonstrated with GlcNAc, as R2YE agar plates containing GlcNAc (0.5 % w/v) blocked development of *S. coelicolor* M145. Here we show that the ‘GlcNAc effect’ is widespread but not universal for streptomycetes, as the effect was observed for most but not all streptomycetes tested so far (Fig. 7). This effect was explained by the model presented previously (Fig. 8) (Rigali *et al.*, 2006): when the extracellular concentration of GlcNAc is high, the components of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) would actively transfer the phosphate group from PEP to GlcNAc, preventing the PTS-mediated control of development-specific proteins, which would therefore be unable to trigger formation of aerial hyphae. Without GlcNAc, the phosphate group from PEP is transferred to one or more as yet unidentified developmental proteins, resulting in development on rich media (such as R2YE agar). This model suggests that GlcNAc possesses at least an equivalent position in the utilization hierarchy to glucose, which is the main carbon source in R2YE agar.

We assessed whether GlcNAc originating from chitin would have the same inhibiting effect on development. In the presence of chitin, GlcNAc is derived from the hydrolysis of chitobiose (notably via the β -N-acetylglucosaminidase DasD) and is phosphorylated via NagK (the GlcNAc kinase) and not by the PTS (Fig. 8). Moreover, as glucose in R2YE exerts a strong carbon catabolite

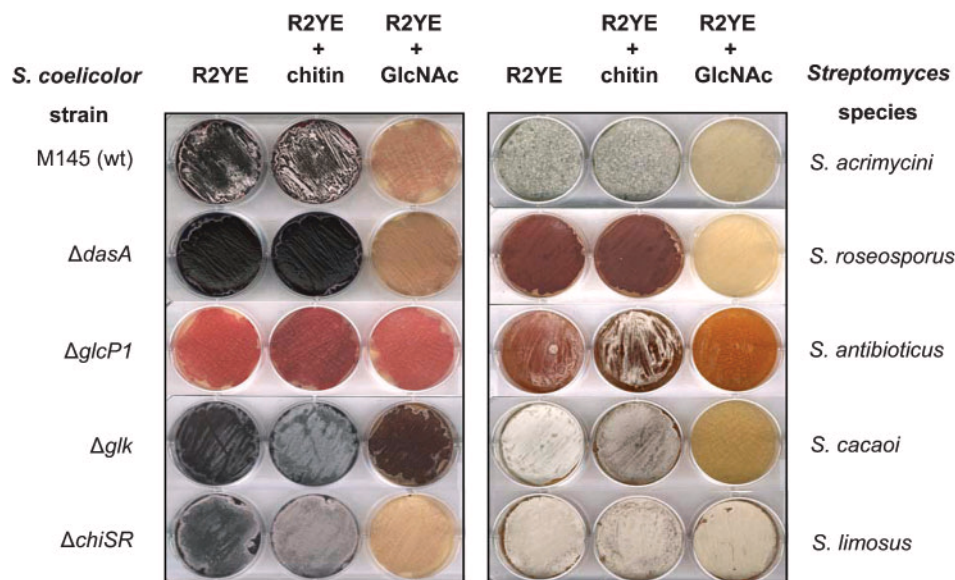


Fig. 7. Effect of GlcNAc and chitin on development in *S. coelicolor* M145 and other streptomycetes on R2YE medium. Null mutants for the glucose transporter (M145 ΔglcP1) and for glucose kinase (M145 Δglk) were used as controls to monitor the effect of chitin and GlcNAc on R2YE plates without the effect of glucose repression. While M145 develops normally on R2YE, concentrations of GlcNAc above 10 mM (20 mM is shown) inhibit development and antibiotic production – particularly that of the blue-pigmented actinorhodin. ΔchiSR and ΔdasA mutants, defective and improved for chitin utilization respectively, were used as negative and positive control to monitor the effect of chitin on morphological differentiation.

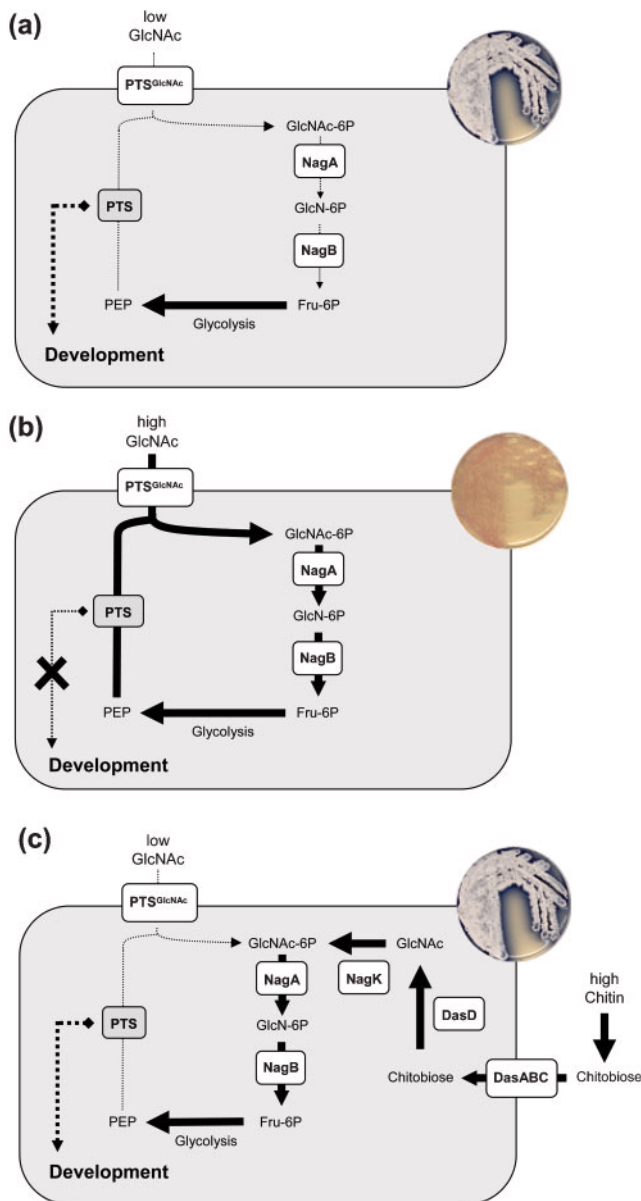


Fig. 8. Effects of addition of GlcNAc or chitin on development of *S. coelicolor* grown in R2YE medium. On R2YE (a), the $\text{PTS}^{\text{GlcNAc}}$ is expressed at its basal level. Under these conditions the intracellular PTS components would transfer the phosphate from PEP to PTS development-specific target proteins. Upon addition of GlcNAc (b), GlcNAc is transported and metabolized to glucosamine 6-phosphate (GlcN-6P) and this then induces the GlcNAc regulon by inhibiting DasR's DNA-binding ability (Rigali *et al.*, 2006). Under these conditions, the PTS components actively transfer the phosphate from PEP to GlcNAc, preventing the PTS-mediated control of development-specific proteins. Upon addition of chitin in R2YE (c), GlcNAc is derived from the hydrolysis of chitobiose (notably via the β -*N*-acetylglucosaminidase DasD) and is phosphorylated via NagK (the GlcNAc kinase) and not by the PTS. The phosphate from PEP is not sequestered by the incoming sugar and PTS protein can target developmental proteins for the phosphotransfer. Abbreviations: NagK, GlcNAc kinase; DasD, β -*N*-acetylglucosaminidase; DasABC, the chitobiose transporter; NagA, GlcNAc-6P deacetylase; NagB, GlcN-6P isomerase.

that in both the presence and the absence of glucose repression (i.e. in M145 and in M145 ΔglkA , respectively) sporulation was normal on R2YE + chitin strongly suggests that the failure of chitin to inhibit or delay development of *S. coelicolor* is not due to glucose repression of the chitinolytic system. Therefore, GlcNAc originating from chitin, which enters the cell not via the PTS but via the PTS-independent DasABC transporter, does not deliver the same information to the developmental programme (Fig. 8). Interestingly, the GlcNAc blocking effect was less severe for mutants affected in glucose repression, i.e. in ΔglkA and ΔglcP1 (Fig. 7), suggesting signalling connections between glucose and GlcNAc-related processes.

DISCUSSION

We show here that the DasABC chitobiose transporter is a second DasR-dependent sugar transporter, in addition to the $\text{PTS}^{\text{GlcNAc}}$, that plays an important role in both primary metabolism and morphological differentiation of *S. coelicolor*. Regarding the role of DasA in primary metabolism, the increased global chitinolytic activity observed for the *dasA* mutant may seem unusual, as inactivation of the chitobiose sensory protein should rather prevent the extracellular sensing of chitin as well as the transport of the chitin-derived inducer of the chitinolytic system. Expression of the chitinolytic genes is rigorously regulated and a plausible explanation for the role of DasA function is inferred from the model of the induction of the chitinolytic system in *Vibrio* species (Li & Roseman, 2004). This model (Fig. 9) comprises three components: (i) the environmental signal, $(\text{GlcNAc})_2$, and possibly larger chitin oligosaccharides; (ii) DasA, the solute-binding protein specific for $(\text{GlcNAc})_n$ ($n > 1$), and its respective ABC-type permeases DasB and DasC; and (iii) the two-component regulatory system ChiS/ChiR. Disruption of *chiR* caused reduced expression of *chiC* in *S. coelicolor* (Kormanec *et al.*,

repression on chitin utilization, the availability of GlcNAc from chitin is significantly delayed. Therefore, as expected, for all streptomycetes tested so far, the addition of chitin into R2YE is not able to block development, and for some strains sporulation is stimulated and/or occurs precociously (Fig. 7). In order to check the 'chitin effect' on development of *S. coelicolor* in strains insensitive to glucose catabolite repression (Angell *et al.*, 1994), we streaked the glucose transporter mutant BAP17 (ΔglcP1), and a new glucose kinase null mutant of *S. coelicolor* (M145 ΔglkA ; B. Traag, S. Colson, S. Rigali & G. P. van Wezel, unpublished) on R2YE, R2YE + chitin and R2YE + GlcNAc. The *glcP1* mutant BAP17 showed a bald phenotype on all media and, interestingly, the 'GlcNAc effect' was lost, as BAP17 was insensitive to a high concentration of GlcNAc, which blocks development of *S. coelicolor* M145 (Fig. 7). The fact

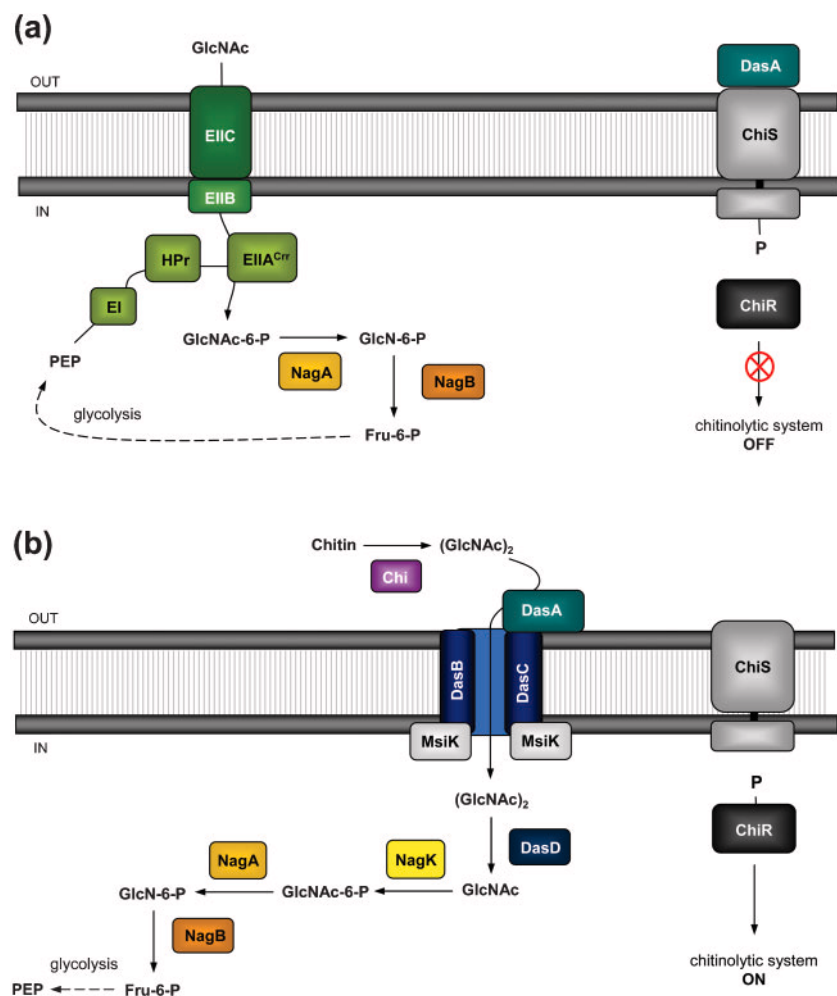


Fig. 9. Model for the regulation of the chitinolytic system in *S. coelicolor*. (a) Repression ('minus' phenotype) and (b) induction ('plus' phenotype) of the chitinolytic system in *S. coelicolor*. In the presence of chitobiose or a related metabolic product of chitin, ChiS is autophosphorylated at a conserved histidine residue (H1199). The phosphoryl group is then transferred to a conserved aspartate (D54) of ChiR, which then binds to the *chi* promoter regions to activate expression of chitinolytic (*chi*) genes. The repression of *chi* genes, the 'minus' phenotype, would be the result of binding of DasA to the extracellular domain of the sensor ChiS, 'locking' it into an inactive conformation. In the presence of chitin, the extracellular signal, typically (GlcNAc)₂, competes with the sensor ChiS for binding to DasA. ChiS could not bind the DasA–(GlcNAc)₂ complex and would thereby be activated to the 'plus' phenotype, followed by the transcriptional activation of the chitinolytic genes by ChiR.

2000). The suggested model predicts that in the presence of chitobiose or a related chitin breakdown product, ChiS is autophosphorylated at a conserved histidine residue (H1199). The phosphoryl group is then transferred to a conserved aspartate (D54) of ChiR, which then binds to the *chi* promoter regions to activate expression of *chi* genes. According to what is proposed in *Vibrio* (Li & Roseman, 2004), the repression of the chitinolytic genes, the 'minus' phenotype, would be the result of the binding of DasA to the extracellular domain of the sensor ChiS, thereby 'locking' it into an inactive conformation (Fig. 9a). In the presence of chitin, the extracellularly generated (GlcNAc)_n or, more specifically, (GlcNAc)₂, competes with the sensory ChiS for DasA. ChiS could not bind the DasA–(GlcNAc)₂ complex and would be thereby activated to the 'plus' phenotype (Fig. 9b), whereafter the chitinolytic genes are expressed via the activation of ChiR. According to this model, the absence of DasA (i.e. in the *dasA* null mutant) would leave the sensor ChiS permanently in its activated form, leading to high and constitutive production of chitinases.

From the developmental point of view, the *dasA* mutant has an extraordinary and unique phenotype in that spore

chains show extensive germination, a phenomenon that has to our knowledge never been described before. This premature germination could well be a secondary effect of the disorder of GlcNAc utilization in *dasA* mutants, and in particular due to a lack of the peptidoglycan precursor molecule glucosamine 6-phosphate. It is easy to see how lower levels of this compound could lead to weakened cell walls and therefore premature germination. It is as yet unclear why the inactivation of the chitobiose transporter causes developmental arrest of *S. coelicolor*, and especially when grown in the absence of chitin or chitobiose. GlcNAc (transported by the PTS) and chitobiose (transported by DasABC) are both degradation products of the GlcNAc polymer chitin. However, in contrast to GlcNAc, chitin does not block development of streptomycetes (Fig. 7). Considering the glucose-dependent developmental defects of *dasA* mutants (Fig. 1), it will be important to see whether glucose utilization is affected. As shown in this paper, deletion of *dasA* causes an increase in the total chitinolytic activity. One possibility is that the absence of DasA simulates the abundance of the alternative rich carbon/nitrogen source chitin in the environment, and, via an unknown mechanism, prevents the utilization of more

readily metabolized carbon sources such as glucose, resulting in poor growth and lack of development on glucose-containing media. We have strong evidence for intimately correlated connections between glucose, GlcNAc and chitin utilization pathways in *S. coelicolor*. For instance, preventing GlcNAc or chitin utilization counteracts the induction of glucose transport (H. Nothhaft & F. Titgemeyer, unpublished data). Additionally, the inactivation of *dasR*, which controls both GlcNAc and chitin utilization, results in enhanced glucose catabolite repression (S. Rigali, G. P. van Wezel & F. Titgemeyer, unpublished data). In our opinion, an insight into the regulatory pathways that govern access to glucose or GlcNAc utilization is pivotal in understanding the complex mechanisms that relay information about the state of primary metabolism to morphogenesis in streptomycetes.

The DasA/DasR/PTS/ChiSR system could well be much more complicated, with additional elements participating in the model. In fact, the bald phenotype of *dasA* mutants is primarily observed in densely populated sections, while well-separated single colonies sporulate relatively normally, suggesting that the phenotype of these mutants is controlled by a quorum-sensing-like mechanism. In Gram-positive bacteria, quorum sensing often involves regulatory proteins belonging to the two-component system group (Kleerebezem *et al.*, 1997). Conceivably, the ChiS/ChiR system predicted to interact with DasA could also target one or more developmental genes whose expression is cell density-dependent.

DasA is not the first example of an ABC transporter solute-binding protein that is also involved in the control of morphological differentiation of streptomycetes. This is exemplified by the *bldK* operon, which encodes an oligopeptide importer responsible for the import of an early extracellular signalling molecule for cell differentiation (Nodwell *et al.*, 1996). The reverse is also known, with deletion of the gene for the phosphate-binding protein PstS accelerating (rather than inhibiting) development of *Streptomyces lividans* (Diaz *et al.*, 2005). Additionally, a study aimed at isolating morphogenesis-dependent ADP-ribosylated proteins exclusively identified ligand-binding elements of ABC transporters in *S. coelicolor* (Sugawara *et al.*, 2002). Post-translational modifications such as ADP-ribosylation can prevent the export of these proteins to the surface and therefore inhibit their function in transport and development. Interestingly, the proteomic analysis of the germination-deficient *crp* mutant (Δ SCO3571) revealed that several ABC transporter solute-binding proteins were sequestered intracellularly, perhaps disturbing the perception of the environmental signals that trigger germination (Piette *et al.*, 2005; Derouaux *et al.*, 2004a, b). Thus, it is becoming increasingly clear that extracellular solute-binding proteins such as DasA play an important role in the perception of nutritional signals that control the onset of key morphological processes.

The relationship between the chitobiose sensor/transporter DasA and development suggests that the deletion of

ABC-type sensors/transporters of other abundant carbon sources like cellobiose (CebE) and xylobiose (BxlE) would lead to similar repercussions on morphological differentiation. The same could apply to other sugar transporters predicted to be part of the DasR regulon; for example, highly significant potential DasR responsive elements have been predicted upstream of SCO4286, SCO6005 and SCO2946, all encoding sugar-binding components of ABC-type transporters (Hiard *et al.*, 2007; Colson *et al.*, 2007). Mutational analysis of these and other sugar transporters is currently under way and the outcome of these experiments should provide more insight into the close link between sugar utilization and the onset of development.

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