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Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*

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Members of the soil-dwelling prokaryotic genus *Streptomyces* produce many secondary metabolites, including antibiotics and anti-tumour agents. Their formation is coupled with the onset of development, which is triggered by the nutrient status of the habitat. We propose the first complete signalling cascade from nutrient sensing to development and antibiotic biosynthesis. We show that a high concentration of *N*-acetylglucosamine—perhaps mimicking the accumulation of *N*-acetylglucosamine after autolytic degradation of the vegetative mycelium—is a major checkpoint for the onset of secondary metabolism. The response is transmitted to antibiotic pathway-specific activators through the pleiotropic transcriptional repressor DasR, the regulon of which also includes all *N*-acetylglucosamine-related catabolic genes. The results allowed us to devise a new strategy for activating pathways for secondary metabolite biosynthesis. Such 'cryptic' pathways are abundant in actinomycete genomes, thereby offering new prospects in the fight against multiple drug-resistant pathogens and cancers.

Keywords: development; nutrient sensing; regulation; secondary metabolism; signalling

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INTRODUCTION

As producers of around two-thirds of all known antibiotics, the soil-dwelling Gram-positive filamentous streptomycetes are a paradigm of secondary metabolite-producing microorganisms (Hopwood, 1999). *Streptomyces coelicolor* A3(2) is the most-studied streptomycete; its complete genome sequence (Bentley *et al*, 2002) and that of *S. avermitilis* (Ikeda *et al*, 2003) each revealed more than 20 'cryptic' (silent) biosynthetic gene clusters for secondary metabolites (Challis & Hopwood, 2003). Although there is a clear temporal correlation between the onset of antibiotic production and development, a detailed understanding of the mechanisms underlying this global control is lacking (Bibb, 2005).

Similar to filamentous fungi, streptomycetes form a mycelium of branching hyphae. On nutrient depletion, aerial hyphae are erected on top of the substrate mycelium and these eventually develop chains of unigenomic spores (Chater & Losick, 1997). Antibiotic production is switched on at a time that corresponds to the early stages of development. What are the control mechanisms that tie chemical differentiation—antibiotic production—to the onset of morphological differentiation? Premature commitment to sporulation means that the opportunity for maximum biomass accumulation is lost, whereas failure to initiate development in time means that the required nutrients become depleted (Gonzalez-Pastor *et al*, 2003; Dworkin & Losick, 2005). The lytic dismantling of the vegetative mycelium in *Streptomyces* colonies, which is necessary to provide nutrients for the build-up of the spore-forming aerial mycelium, is a striking example of programmed cell death (Fernandez & Sanchez, 2002; Manteca *et al*, 2006). In eukaryotes, the programmed removal of cells during development or for eradicating defective cells is well studied (Baumann *et al*, 2002; van Loo *et al*, 2002), but it has only recently been recognized as important in bacteria (Rice & Bayles, 2003). The suggestion that antibiotic production is controlled by an apoptosis-like mechanism is reinforced by the failure of most non-developing mutants to produce antibiotics (Bibb, 2005). The remarkable coincidence between the onset of development and of secondary metabolism suggests the presence of a master switch

superimposed on the pathway-specific control mechanisms. Here, we describe the first, to our knowledge, complete signalling cascade from the perception of the nutritional status of the environment to the onset of antibiotic production, which revolves around the global regulatory protein DasR.

RESULTS AND DISCUSSION

N-acetylglucosamine as a signal for antibiotic production

The carbon and nitrogen source *N*-acetylglucosamine (GlcNAc), which is part of the bacterial peptidoglycan and the monomer of the abundant natural polymer chitin, causes developmental arrest of *S. coelicolor* on rich media (Rigali *et al*, 2006). This contrasts with the comparable carbon and nitrogen source glutamate, which as a carbon source is preferred to glucose, but has no apparent effect on development (van Wezel *et al*, 2006). As antibiotic production coincides with morphogenesis, we investigated the effect of GlcNAc on the production of the red-pigmented antibiotic undecylprodigiosin (Red) and the blue-pigmented actinorhodin (Act) of *S. coelicolor* grown on rich (R2YE) and minimal medium (MM) agar plates. The *act* and *red* biosynthetic clusters are transcriptionally activated by RedD/RedZ and ActII-ORF4, respectively (Bibb, 2005). Interestingly, as shown in Fig 1, GlcNAc blocks development and antibiotic production under rich growth conditions (R2YE agar + 10 mM GlcNAc or higher), whereas it triggers Act and Red production and sporulation under poor nutritional conditions (MM agar; 5 mM GlcNAc or higher).

To assess whether the effect of GlcNAc on antibiotic production is widespread among streptomycetes, several *Streptomyces* species were plated onto MM with mannitol (25 mM) as the sole carbon source and with or without GlcNAc (50 mM), using *Bacillus subtilis* as the indicator strain (Fig 2). GlcNAc had a stimulating effect on antibiotic production by the *Streptomyces* species *S. clavuligerus*, *S. collinus*, *S. griseus*, *S. hygroscopicus* and *S. venezuelae*, whereas there was no effect on visible antibiotic production by *S. acrimycini*, *S. avermitilis*, *S. cinnamonensis*, *S. limosus* and *S. rimosus*. An inhibitory effect was observed only for *S. roseosporus*. This suggests that the antibiotic-triggering effect of GlcNAc is common in streptomycetes, although not universal, at least under the conditions we studied.

DasR as a master switch for antibiotic production

In *S. coelicolor*, GlcNAc is internalized and phosphorylated by the sugar phosphotransferase system (PTS; Nothaft *et al*, 2003). The GlcNAc regulon is controlled by the GntR regulator DasR (Rigali *et al*, 2002, 2004), the DNA-binding activity of which is inhibited by glucosamine-6-phosphate (Rigali *et al*, 2006). Interestingly, the *S. coelicolor dasR* mutant BAP29 showed enhanced and accelerated production of pigmented antibiotics, suggesting a role for DasR in the control of antibiotic production (supplementary Fig S1 online). The relative increase in antibiotic production in the *dasR* mutant was quantified by spectrophotometry, and showed that production of Act and Red was consistently enhanced in BAP29 by factors of 3.2 (± 0.2) and 3.9 (± 0.3), respectively.

To identify the precise DasR-responsive element (*dre*), we performed DNase I footprinting on the promoter region of the *crr-ptsI* operon, encoding the PTS enzyme IIA (IIA^{Crr}) and enzyme I (EI; Fig 3A). The protected sequence (TGTGGTCTAGACCTCT) corresponded to positions −130 to −115 relative to the start of *crr*, and had a 13- out of 16-bp match to the *in silico*-predicted consensus DasR-binding site (Colson *et al*, 2007). This information was used to determine the precise *dre* sites of five known target genes. By using this training set, we built a position weight matrix to scan the genome of *S. coelicolor* with the PREDetector program for putative new *dre* sites (supplementary Table S1 online; Rigali *et al*, 2004; Hiard *et al*, 2007). Interestingly, PREDetector identified putative *dre* boxes upstream from *act*II-ORF4 and *redZ*, encoding transcriptional activators of the *act* and *red* gene clusters, respectively (supplementary Table S2 online). The *dre* site upstream from *act*II-ORF4 is located between the −35 and −10 sequences of the promoter, whereas that of *redZ* is situated about 50 bp upstream from the −35 sequence of the *redZ* promoter.

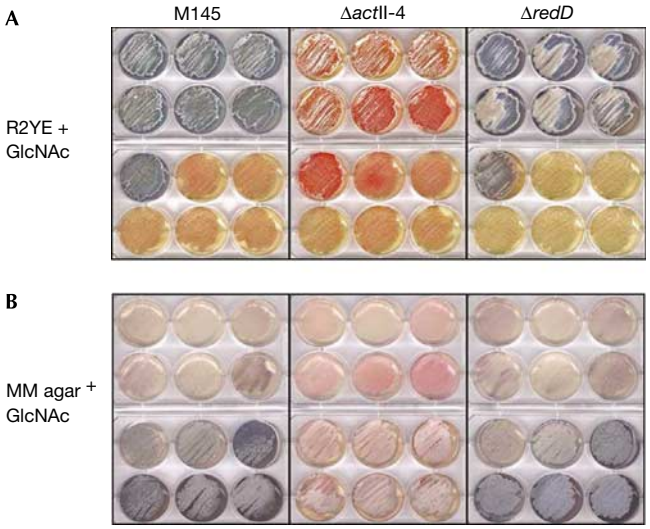

Fig 1 | The dual GlcNAc signal depends on media conditions. On nutrient-rich R2YE plates (**A**), GlcNAc blocks morphogenesis and antibiotic production, whereas on nutrient-depleted MM agar plates (**B**), GlcNAc has the opposite effect. GlcNAc concentrations (left to right, top to bottom) are 0, 0.001, 0.01, 0.1, 1, 5, 10, 20, 50, 100, 150 and 200 mM. The effect of GlcNAc on each antibiotic was assessed using the *act*II-ORF4 and *redD* null mutants for monitoring the production of undecylprodigiosin and actinorhodin, respectively. GlcNAc, *N*-acetylglucosamine; MM, minimal medium.

Electrophoretic mobility gel shift assays (EMSAs) with purified His_6-tagged DasR on ^{32}P-radiolabelled DNA probes (in the presence of a 100-fold molar excess of unlabelled φX174 DNA) encompassing the promoter regions of *act*II-ORF4, *redZ* or *ptsH* (positive control, encoding the PTS HPr protein) showed direct binding of DasR to all three DNA fragments, with a single large conformational shift for *act*II-ORF4, redZ and two binding sites for *ptsH*, which correspond to the two known *dre* sites (Fig 3B). Use of double-stranded oligonucleotide probes containing only the *dre* elements with short flanking sequences showed that DasR bound to the *dre* elements for the *act*II-ORF4, *redZ* and *crr-ptsI* promoter regions, but not to the *cis*-acting element of Crp (negative control; Fig 3B).

Semiquantitative reverse transcription–PCR (RT–PCR) analyses further showed upregulation of *act*II-ORF4 and *redZ* in the *dasR* mutant (Fig 3C). Enhanced expression of *redZ* strongly induces transcription of the red pathway-specific activator gene *redD* (Guthrie *et al*, 1998), and *redD* was also upregulated in the mutant.

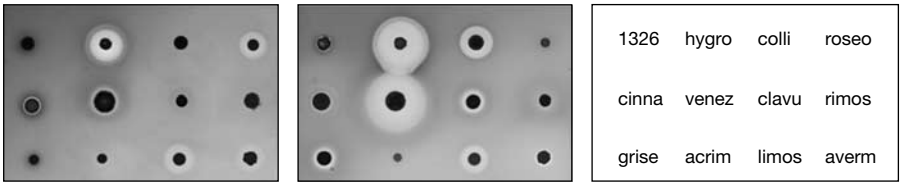

Fig 2 | Conservation of the GlcNAc-dependent antibiotic-inducing pathway among streptomycetes. Streptomycetes were grown on minimal medium agar plates with 25 mM mannitol alone (left panel) or with the addition of 50 mM GlcNAc (middle panel). *Bacillus subtilis* was used as an indicator strain. For strains (right panel), see Table 1. GlcNAc triggered antibiotic activity in *S. hygroscopicus*, *S. collinus*, *S. venezuelae*, *S. clavuligerus* and *S. griseus*, whereas it inhibited antibiotic production in *S. roseosporus*. GlcNAc, *N*-acetylglucosamine.

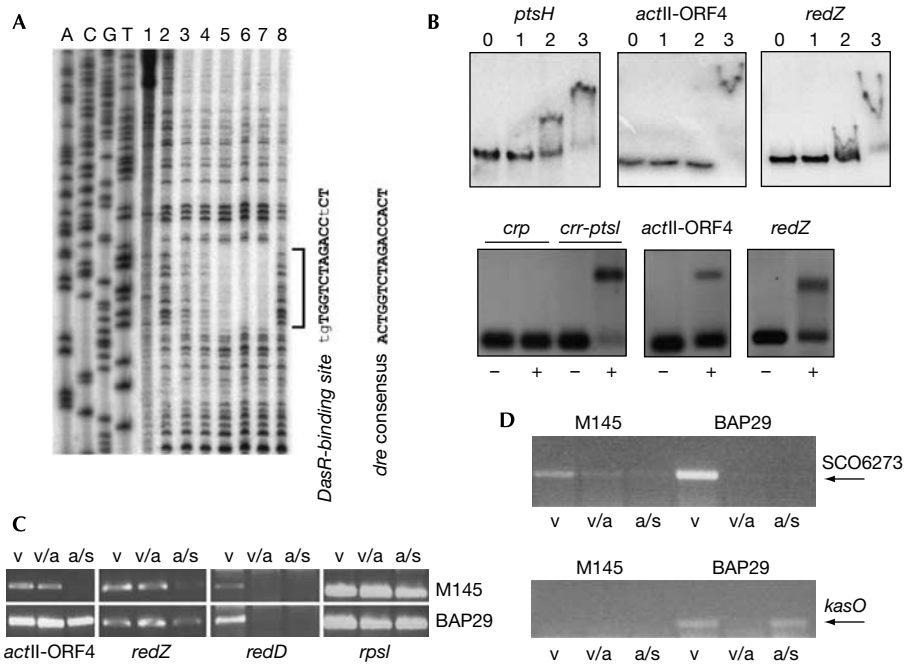

Fig 3 | DasR controls antibiotic production in *Streptomyces coelicolor*. (**A**) Identification of the DasR-binding site by DNase I footprint analysis of the *crr-ptsI* upstream region. The *crr-ptsI* probe was incubated with DNase I (0.4 μg/ml) and increasing amounts of purified DasR (0, 10, 20, 40, 60 or 80 pmol of DasR in lanes 2, 3, 4, 5, 6 and 7, respectively). Controls: lane 1, no DasR or DNase I; lane 8, DNase I and 350 pmol of bovine serum albumin. ACGT, DNA sequence lanes. (**B**) Electrophoretic mobility gel shift assays showing direct interaction of DasR with *dre* sites predicted upstream from *act*II-ORF4 and *redZ*. Top: binding of DasR to the entire promoter regions of *ptsH*, *act*II-ORF4 and *redZ*; lane 0, probe; lanes 1, 2 and 3, probe and 1, 5 and 20 nM DasR, respectively; bottom: double-stranded oligonucleotide probes encompassing *dre* sites for *crr-ptsI* (positive control), *act*II-ORF4 and *redZ* were incubated with (+) or without (−) purified His-tagged DasR. The *crp* promoter region was used as a negative control. (**C**) Transcription of the pathway-specific activator genes for Act (*act*II-ORF4) and Red (*redD*) analysed by semiquantitative RT–PCR. Samples were collected from *S. coelicolor* M145 and the *dasR* mutant BAP29 grown on minimal medium mannitol plates after 30 h (vegetative growth (v)), 42 h (initiation of aerial growth (a)) and 72 h (aerial growth and spores (s)). *rpsI* (for ribosomal protein S9) was used as an RNA integrity control. (**D**) Transcriptional analysis of the *kas* 'cryptic' type I polyketide cluster of *S. coelicolor* (SCO6273–6288) by semiquantitative RT–PCR. Transcriptional repression of *kasO* is relieved by deletion of *dasR*. RNA samples are as in (**C**). RT–PCR, reverse transcription–PCR.

Hence, the activator genes of the *act* and *red* clusters are downregulated by DasR, explaining the enhanced production of the respective antibiotics in the *dasR* mutant. A signalling cascade is thus described from nutrient sensing to antibiotic production (Fig 4).

A major question concerns the origin of the GlcNAc-derived signal. Previously, we reported a marked difference in the effects of either GlcNAc or its polymeric form, chitin, on differentiation, as GlcNAc blocks development and antibiotic production under nutrient-rich conditions, whereas chitin has no effect (Colson *et al*, 2008). This suggests that the cell can distinguish between chitin-derived GlcNAc (signalling abundance) and GlcNAc derived from cell-wall lysis (signalling starvation). According to our bioinformatic predictions, chitinase genes and the gene for D-Ala-D-Ala aminopeptidase (DppA, SCO6489), responsible for catabolism of the cell-wall precursor D-Ala-D-Ala under nutrient deficiency (Cheggour *et al*, 2000), are part of the DasR regulon. To explain

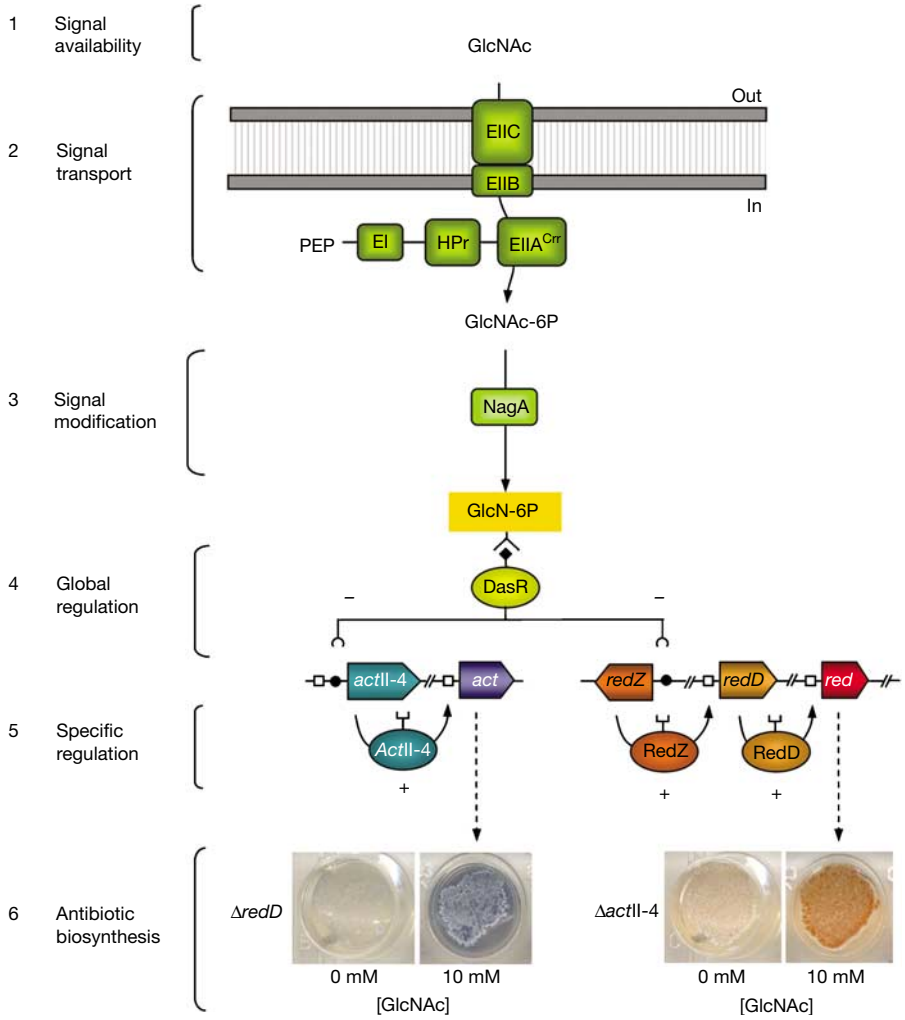

Fig 4 | Model of GlcNAc-dependent signalling in streptomycetes. GlcNAc enters the cytoplasm and is phosphorylated by the PTS, which is composed of intracellular general PTS proteins EI, HPr and EIIA, and the GlcNAc-specific EIIB and EIIC components. *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) is deacetylated by NagA. The resulting glucosamine-6-phosphate (GlcN-6P) is an allosteric effecter of DasR that inhibits its DNA binding, resulting in the loss of transcriptional repression of *act*II-ORF4 and *redZ*, and thereby activating actinorhodin (Act) and undecylprodigiosin (Red) production, respectively. The stimulatory effect of GlcNAc on antibiotic production is visualized by using the *S. coelicolor* mutants M511 (Act non-producer) and M510 (Red non-producer), respectively. E, enzyme; GlcNAc, *N*-acetylglucosamine; NagA, *N*-acetylglucosamine deacetylase; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

this, we measured the overall chitinase and DppA activities. Under conditions in which antibiotics are overproduced in the *dasR* mutant (that is, on MM), there was a fivefold higher activity of DppA, whereas at the same time BAP29 lost substrate induction of the chitinolytic system by chitin (S.R., F.T., S.B., S.M., A.W.T., D.A.H. & G.P.v.W., unpublished data). This suggests coordination between antibiotic production and cell-wall lysis in a DasR-dependent manner (see below).

Awakening cryptic clusters

Understanding the control of cryptic biosynthetic clusters is a major challenge in modern antibiotic research (Van Lanen & Shen, 2006; Wilkinson & Micklefield, 2007). We analysed the role of DasR in the control of a biosynthetic gene cluster for a hypothetical antibiotic made by a type I modular polyketide synthase (SCO6273–6288), the induction of which depends on *kasO* (SCO6280; Takano *et al*, 2005). *kasO* transcripts were identified in RNA from early and late samples of BAP29, but not in wild-type samples (Fig 3D). Most probably as a result of the induction of *kasO*, transcription of SCO6273, the last open reading frame of the cryptic cluster, was markedly increased during vegetative growth (Fig 3D). No *dre* consensus sequence was found upstream from *kasO*, and the manner in which the *kas* gene cluster is controlled by DasR is now under investigation in our laboratories.

Speculation

The signalling function of GlcNAc should depend on environmental conditions: development is undesirable under nutrient-rich ('feast') conditions, but should be triggered under 'famine'

conditions. Therefore, the decision depends on the availability of the two principal sources of GlcNAc in nature: chitin and the bacterium's own cell wall. Both chitinases and DppA are part of the DasR regulon. Both antibiotic production and hydrolysis of cell-wall precursors by the D-Ala-D-Ala aminopeptidase seem to be strongly enhanced in the *dasR* mutant, while this strain has lost its ability to induce the chitinolytic system. Thus, in a situation in which DasR is inactive and antibiotic production is switched on, chitin degradation is repressed while the cell activates catalytic enzymes that break down peptidoglycan building blocks. This supports our hypothesis that the accumulation of GlcNAc during cell-wall hydrolysis (famine) triggers development and antibiotic production, whereas utilization of chitin-derived GlcNAc (feast) should block development. This fine-tuning allows the organism to recruit an omnipresent compound as a signalling molecule, and to decide between growth and commitment to the irreversible final lifecycle step of sporulation.

Note added in proof

Recently, McArthur and Bibb used an elegant technique to scan the *act*II-ORF4 promoter region for new regulatory elements and to monitor their effects *in vivo* (McArthur & Bibb, 2008). This highlighted a regulatory element located between the −35 and −10 consensus sequences of the *act*II-ORF4 promoter that should be a target for a transcriptional repressor. This regulatory element is in fact the *dre* identified in this work, providing further *in vivo* substantiation for the direct repressing activity of DasR on actinorhodin production.

METHODS

Bacterial strains and culture conditions. The species of *Streptomyces* used in this work are shown in Table 1. All media and routine *Streptomyces* techniques have been described previously (Kieser *et al*, 2000). Phenotypic characterization of mutants was carried out on MM agar plates (Kieser *et al*, 2000) with carbon sources as indicated. Quantification of Act and Red was performed as described previously (Martinez-Costa *et al*, 1996).

DNase I footprinting. A 211 bp PCR-amplified DNA fragment corresponding to the −202–+8 region relative to the start of *S. coelicolor crr* (SCO1390) was chosen for DNase I footprinting. A 50 fmol portion of ^{32}P-end-labelled probe was incubated with the relevant proteins (DasR-His$_6$ and/or BSA) and DNase I (0.4 μg/ml) as described previously (Sambrook *et al*, 1989).

Electrophoretic mobility gel shift assay. For EMSAs on the entire promoter regions, we used 1–20 nM of His$_6$-tagged DasR and 1 nM of ^{32}P-end-labelled PCR-amplified DNA probes, whereas EMSAs on double-stranded oligonucleotides were performed using 50 nM of non-radiolabelled probes and 200 nM of His-tagged DasR. All the reactions were carried out in binding buffer (20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl and 10% glycerol) containing 100 μg/ml bovine serum albumin and a 100- or 10-fold molar excess of *Hpa*II-digested φX174 DNA for EMSAs on PCR fragments and oligonucleotides, respectively. Promoter fragments were the −134–+97 fragment of *act*II-ORF4, the −280–+78 fragment of *redZ* and the −249–+103 fragment of *ptsH* (nucleotide position relative to the translational start sites), and double-stranded oligonucleotide probes encompassing the predicted *dre* elements were taken from the promoter regions of *act*II-ORF4 (SCO5085), *redZ* (SCO5881) and *crr* (SCO1390); the *cis*-acting element of *crp* (SCO3571; not controlled by DasR) was used as a negative control. For oligonucleotides, see the supplementary Table S3 online.

Table 1 | *Streptomyces* species used in this work

***Streptomyces* species**	**Genotype**	**Reference or source**
S. coelicolor M145	WT	JIC
S. coelicolor BAP29	Δ*dasR*	Rigali *et al* (2006)
S. coelicolor M510	Δ*act*II-ORF4	Floriano & Bibb (1996)
S. coelicolor M511	Δ*redD*	Floriano & Bibb (1996)
S. coelicolor M512	Δ*act*II-ORF4 Δ*redD*	Floriano & Bibb (1996)
*S. **acrim**ycini* DSM 40540	WT	DSMZ
*S. **averm**itilis* NRRL 8165	WT	ATCC
*S. **cinna**monensis* DSM 40467	WT	DSMZ
*S. **clavu**ligerus* NRRL 358	WT	DSMZ
*S. **colli**nus* DSM 40733	WT	DSMZ
*S. **griseus*** NRRL B2682	WT	DSMZ
*S. **hygro**scopicus* ATCC27438	WT	ATCC
*S. **limosus*** ATCC 19778	WT	ATCC
S. lividans **1326**	WT	JIC
*S. **rimosus*** ATCC 10970	WT	ATCC
*S. **roseo**sporus* ATCC 31568	WT	ATCC
*S. **venez**uelae* ATCC15439	WT	ATCC

The parts of the species names shown in bold refer to the abbreviations used in Fig 2. ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen; JIC, John Innes Centre Strain collection; WT, wild type.

Reverse transcription–PCR. RNA was isolated from the mycelium of *S. coelicolor* M145 and BAP29 collected after 30 h (vegetative growth), 42 h (initiation of aerial growth) or 72 h (aerial growth and spores) from MM mannitol plates with cellophane discs (grown at 28 °C). RT–PCR analyses were conducted using the Superscript III one-step RT–PCR Kit (Invitrogen, Carlsbad, CA, USA). RT–PCRs without reverse transcription were used as a control for the 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 (van Wezel *et al*, 2005). Data were verified in three independent experiments. For oligonucleotides, see supplementary Table S3 online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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