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DasR is a pleiotropic regulator required for antibiotic production, pigment biosynthesis, and morphological development in *Saccharopolyspora erythraea*

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Abstract The GntR-family transcription regulator, DasR, was previously identified as pleiotropic, controlling the primary amino sugar *N*-acetylglucosamine (GlcNAc) and chitin metabolism in *Saccharopolyspora erythraea* and *Streptomyces coelicolor*. Due to the remarkable regulatory impact of DasR on antibiotic production and development in the model strain of *S. coelicolor*, we here identified and characterized the role of DasR to secondary metabolite production and morphological development in industrial erythromycin-producing *S. erythraea*. The physiological studies have shown that a constructed deletion of *dasR* in *S. erythraea* resulted in antibiotic, pigment, and aerial hyphae production deficit in a nutrient-rich condition. DNA microarray assay, combined with quantitative real-time reverse transcription PCR (qRT-PCR), confirmed these results by showing the downregulation of the genes relating to secondary metabolite production in the *dasR* null mutant. Notably, electrophoretic mobility shift assays (EMSA) showed DasR as being the first identified regulator that directly regulates the pigment biosynthesis *rpp* gene cluster. In addition, further studies indicated that GlcNAc, the major nutrient signal of DasR-responsed regulation, blocked secondary metabolite production and morphological development. The effects of GlcNAc were shown to be caused by DasR mediation. These findings demonstrated that DasR is an important pleiotropic regulator for both secondary metabolism and morphological development in *S. erythraea*, providing new insights for the genetic engineering of *S. erythraea* with increased erythromycin production.

Keywords *Saccharopolyspora erythraea* · DasR · Secondary metabolism · Morphological development

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Introduction

Saccharopolyspora erythraea is a Gram-positive actinomycete that is used industrially for the production of erythromycin A (Staunton and Wilkinson 1997), an important broad spectrum macrolide antibiotic against pathogenic Gram-positive bacteria. The derivatives of erythromycin also play a vital role in medicine. Given its industrial importance, extensive efforts have been made to understand the regulatory mechanism of erythromycin biosynthesis in *S. erythraea*. One such effort has been the deciphering of its genomic information by complete genome sequencing (Oliynyk et al. 2007), which has facilitated advancement in strain improvement, and genetic and metabolic engineering. The factors that affect erythromycin production are complex, one of which is the direct regulation of the erythromycin biosynthesis (*ery*) cluster through transcription factors. However, a significant discrepancy between the *ery* cluster and other Streptomyces antibiotic biosynthesis clusters is that the *ery* cluster does not contain cluster-situated regulator (CSR) genes (Bibb 2005; Reeves et al. 1999). Recently, BldD, a key developmental regulator in actinomycetes (Elliot et al. 1998; Elliot et al. 2001), was

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identified to directly bind and positively and transcriptionally regulate the *ery* cluster, as well as affect the morphogenetic program in *S. erythraea* (Chng et al. 2008). Additionally, several studies have made further contribution in uncovering the regulatory mechanism of secondary metabolite production in *S. erythraea* (Kirm et al. 2013; Mironov et al. 2004; Wu et al. 2014; Yao et al. 2014).

Apart from the direct regulation of erythromycin biosynthetic genes, transcript levels of the genes involved in the metabolism of the precursors (propionyl-CoA and methylmalonyl-CoA) of erythromycin biosynthesis also have a significant effect on erythromycin yield (Li et al. 2013). These include biotin-dependent carboxylases that catalyze the carboxylation of propionyl-CoA to (2*S*)-methylmalonyl-CoA, encoded by at least five genetic loci; adenosylcobalamine-dependent methylmalonyl-CoA mutase catalyzes the reversible conversion of succinyl-CoA and (2*R*)-methylmalonyl-CoA, and methylmalonyl-CoA epimerase interconverts (2*R*)- and (2*S*)-isomers (Oliynyk et al. 2007). In addition, some primary metabolic pathways also greatly affected the flux of metabolites through the erythromycin feeder pathways. For instance, the valine catabolic pathway was reported to be a major feeder pathway of erythromycin biosynthesis (Carata et al. 2009).

Recently, we characterized the GntR family protein, DasR, in *S. erythraea* (Liao et al. 2014a) as playing a similar regulatory role in the primary chitin and *N*-acetylglucosamine (GlcNAc) metabolism previously identified in *Streptomyces coelicolor* (Colson et al. 2007; Rigali et al. 2006). By these efforts, a consensus binding site for DasR (*dre*, for the DasR-responsive element) has been derived from its identified target genes (Colson et al. 2007; Liao et al. 2014a). Remarkably, the DasR regulon uncovered a range of targets relating to antibiotic biosynthesis, revealing DasR as a pleiotropic regulator of secondary metabolism in *S. coelicolor*. In fact, DasR regulates antibiotic biosynthesis by directly repressing the transcriptional activity of CSR genes *actII-ORF4* and *redZ*, the transcriptional activators of the actinorhodin (Fernandez-Moreno et al. 1991) and undecylprodigiosin (Narva and Feitelson 1990) biosynthetic gene clusters. Moreover, DasR also plays a significant role in the development of *S. coelicolor*, as *dasR* null mutants led to a bald phenotype on glucose-containing media (Rigali et al. 2006). Interestingly, GlcNAc also affected the antibiotic production and development of *S. coelicolor*, depending on the nutrient condition, namely GlcNAc blocked development and antibiotic production when cultured on rich media, while these processes were promoted on minimal media (Rigali et al. 2008). In fact, the assimilation of GlcNAc is the premise that triggers DasR-mediated transcriptional regulation (Nothaft et al. 2010). Additionally, glucosamine-6-phosphate (GlcN-6-P), the metabolic intermediate of GlcNAc, allosterically changes the binding activity of DasR toward its target promoters (Rigali et al. 2006). Although an integrated signaling cascade formed by the external nutrient (GlcNAc) and the DasR-mediated response in the regulation of antibiotic production and development was elucidated in *S. coelicolor*, an equivalent mechanism mediated by DasR in other industrial antibiotic-producing actinomycetes remains unknown.

Here, we aim to investigate the role of DasR in linking GlcNAc signals to secondary metabolite production and morphogenesis in *S. erythraea* as well as to discuss its common and distinct features in reference to *S. coelicolor*. In this study, the deletion of *dasR* led to decreased antibiotic pigment biosynthesis and delayed development of *S. erythraea* in nutrient-rich conditions. Through transcriptome and transcription analysis, in combination with gel retardation assay, the molecular mechanism was revealed, suggesting that DasR directly or indirectly modulates many important genes relating to secondary metabolism. This study highlights the crucial role of DasR in the regulation of secondary metabolism and development in industrial antibiotic-producing actinomycetes.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. erythraea* strains were grown on R2YE agar plates, as described in *Practical Streptomyces Genetics* (Kieser et al. 2000), at 30 °C for sporulation. An agar sample of about 1 cm^2 was cut and inoculated into a 250-ml flask containing 25 ml of Tryptic Soy Broth (TSB) and grown for 48 h at 30 °C and 200 rpm for seed-stock preparation. Then, 0.5 ml of the seed cultures was inoculated in a 500-ml flask containing 50 ml TSB medium, in the same culture conditions, and the cell samples were harvested at the indicated time points for RNA extraction. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. All media types were sterilized by autoclaving at 121 °C for 20 min. GlcNAc (A3286) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Phenotypic analysis

S. erythraea strains were grown on R2YE agar plates, covered with plastic cellophane, at 30 °C. For dry weight biomass determination, cultures were harvested at the indicated time points and then dried overnight at 60 °C. Erythromycin production was measured by using a bioassay, as described previously (Chng et al. 2008), with modification. *Bacillus subtilis* was used as an indicator strain. A standard concentration gradient of erythromycin A was used to quantify the erythromycin titer diffused in the agar. The method for pigment measurement was described previously (Magdevska et al. 2010).

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference
Strains		
S. erythraea		
NRRL23338	Wild-type *S. erythraea* strain	DSM 40517
Δ*dasR*	*dasR* null mutant strain, thiostrepton resistance	This study
E. coli		
DH5α	Recipient for cloning experiments	GIBCO-BRL
BL21(DE3)	F-*ompT hsdS gal dcm* (DE3)	Novagen
Plasmids		
pET28a	Expression vector, Kan^r	Novagen
pET-*dasR*	pET28a derivative carrying *dasR* of *S. erythraea*	This work
pMD-18 T	TA-cloning vector	Takara
pUC18-*tsr*	pUC18 derivative containing a 1.36-kb fragment of a thiostrepton resistance cassette in the *Bam*HI/*Sma*I sites	(Han et al. 2011)
pUC-*dasR*	pUC18-*tsr*, with the 1.5-kb DNA fragments upstream and downstream of *dasR* inserted upstream and downstream of *tsr*.	This study

The soluble flaviolin was extracted by methanol from an equal amount of agar samples. The concentration of pigment was converted to optical density (OD) at 270 nm, measured by a microplate reader (BioTek, USA).

Overexpression and purification of DasR protein

To express the DasR protein, the gene of *dasR* (SACE_0500/NC_009142_0499) was amplified by PCR from *S. erythraea* NRRL23338 genomic DNA, using the primers DasR-fw/rev (Table 2), and was then cloned into pET-28a(+), generating the recombinant plasmid pET-*dasR*. After DNA sequencing confirmation, the recombinant plasmids were introduced into *E. coli* BL21 (DE3). The *E. coli* cells were grown in 50 ml LB medium at 37 °C with 25 mg ml^{-1} kanamycin in an orbital shaker (250 rpm, 37 °C) to an OD_{600} of 0.6. Expression was then induced with IPTG at a final concentration of 0.5 mM, followed by incubation at 20 °C for 6–8 h. For protein purification, cells were harvested by centrifugation and washed twice with PBS buffer (pH 8.0) and then broken by an ultrasonic cell crusher. Cell debris and membrane fractions were separated from the soluble fractions by centrifugation (45 min, 15,000 rpm, 4 °C). His_6-DasR was purified by Ni-NTA Superflow columns (Qiagen). Proteins were eluted with 250 mM imidazole (in 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The purified proteins were dialyzed in protein preservation Buffer D (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, 20 % glycerol, 1 mM DTT, pH 8.0) at 4 °C overnight and then stored at −80 °C until use. The quality of the purified proteins was determined by SDS-PAGE. Protein concentration was determined by the Bradford reagent.

Construction of the dasR in-frame deletion mutant

The *dasR* gene mutation strategy was described previously (Liao et al. 2014a; Liao et al. 2014b). In order to construct an in-frame deletion of 756 nt (GenBank accession no. NC_009142.1 553254.554009), a fragment of the SACE_0500 gene, two 1.5-kb DNA fragments of the adjacent region were amplified from the *S. erythraea* NRRL23338 genomic DNA by PCR, using the primer pairs *dasR*-up-fw/rev (upstream fragment) and *dasR*-dw-fw/rev (downstream fragment) (Table 2), then the PCR products were digested with *Eco*RI/*Bam*HI and *Kpn*I/*Hin*dIII, respectively, and subsequently inserted into the corresponding sites of pUC18-*tsr*, creating the pUC-*dasR* knockout plasmid. The resulting mutant plasmid was transferred into *S. erythraea* NRRL23338 by polyethylene glycol (PEG)-mediated transformation. For the linear fragment homologous recombination strategy, the *dasR* gene in *S. erythraea* was replaced with the thiostrepton resistance gene cassette. The selected mutants were verified by PCR and DNA sequencing.

Electrophoretic mobility shift assay

The entire promoter region of the *rpp* gene cluster was amplified by PCR with gene-specific primers containing a universal primer (5′-AGCCAGTGGCGATAAG-3′) sequence (Table 2) and biotin labeled by PCR with the 5′ biotin-labeled universal primer. The PCR products were identified by agarose gel electrophoresis and purified by the PCR Purification Kit (Shanghai Generay Biotech Co., Ltd.). The concentrations of biotin-labeled DNA probes were determined with a microplate reader (Biotek, USA). Electrophoretic mobility shift

Table 2 The oligonucleotides used in this study

Oligonucleotides	Sequence (5′–3′)
Construction of the pET-*dasR* plasmid for DasR overexpression	
DasR-fw	CATGCCATGGTCGAAACATCGGTGCCA
DasR-rev	CCCAAGCTTGGCGGGCGGGTTGAGG
Construction of the *dasR* knockout mutant strain	
dasR-up-fw	AACTGCAGGTCTCGGCCAGCTTCAGGG
dasR-up-rev	GCGGGATCCCGTTCCTCCTACCACCGCAA
dasR-dw-fw	TATGGTACCTACAAGTTCGTCGCCCGC
dasR-dw-rev	CCGGAATTCCACCGACCTCAGCGTTTACG
Primers for *dasR* mutant confirmation by DNA sequencing	
Up-*dasR*	GGCTGGCTCGGGTGGAT
Up-*tsr*	GAGTTGCTGGATCTGTGCG
Dw-*tsr*	ACGACGGGAAGGGAGAAG
Dw-*dasR*	CATGGCTCCCACGCACTCG
Primers for PCR amplification of EMSAs probe with biotin labeling	
Universal primer	^{Biotin}-AGCCAGTGGCGATAAG
UR^{rppA}-fw	AGCCAGTGGCGATAAGCTCCGCC TGAAACAG
UR^{rppA}-rev	AGCCAGTGGCGATAAGGCATAGAA CTGCCACC
Primers for real-time RT-PCR	
P5638-fw	CACCTACGACGGCATCA
P5638-rev	GATCTCGCGGTTGGTCT
P5639-fw	GCGCACAACCTGCTGA
P5639-rev	TCCTGGCCCATCTTGG
P5640-fw	GTGCTCGTGGAAACGG
P5640-rev	GATGCCCTGCAACTGG
P6238-fw	AGGTCGCCCACATCG
P6238-rev	AGTCGTCCCGCATCG
P1456-fw	CTTGGGCACTTCATCGG
P1456-rev	CGCTTCTGCGGGTTCT
P1457-fw	TCCAAGCTCAGCGACTT
P1457-rev	CGGCAAGGTATCCGTAA
P1458-fw	AGCGAGACGTTGTGCC
P1458-rev	ATGCCGATCTTGGAGC
P1459-fw	GGAGTGCTACGACCAGG
P1459-rev	GCCATCGGCTCAAGAA
P1241-fw	ACGCCTTCGCCAACT
P1241-rev	ACGCAGGTCTTCCACC
P1242-fw	CCGCAACAACAGCACG
P1242-rev	CGGCGATGTAGGAGACG
P1243-fw	CTACCGCTCGTCCTTCG
P1243-rev	TGGCCTGCTCCTCGTA
P1093-fw	GAAGCCGTCGGCACTCT
P1093-rev	GGCATTCCGCCCTGAT
P2141-fw	GCTCGCTGACCGAACCA
P2141-rev	CGGGACTTCGCCTGGAT
P2853-fw	GCGACACGACGAACTGG
P2853-rev	TCATCCCACCGAAGACG
P5583-fw	TTCCACCCCGACCAC
P5583-rev	TCAGACCGCCCCAGAC
P6426-fw	CGAGGACCCGGAACTGT
P6426-rev	AGGCACTCGGAGGCAAC
P6464-fw	CAGGAATGGCAGGAGC
P6464-rev	CGTGTTCGAGGGCG TA
P2077-fw	GGTCGTCGGGTCCTAT
P2077-rev	GCACCTTGCCGTTGT

assay (EMSA) was carried out according to the protocol accompanying the Chemiluminescent EMSA Kit (Beyotime Biotechnology, China). The binding reaction contained 10 mM Tris HCl (pH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.01 % Nonidet P40, 50 μg ml^{-1} poly[d(I-C)], and 10 % glycerol. After binding, the samples were separated on a non-denaturing PAGE gel in an ice bath of 0.5× Tris-borate-EDTA at 100 V and bands were detected by BeyoECL Plus (Beyotime Biotechnology, China).

Transcriptome assay

The *S. erythraea* DNA microarrays (SER v1.0) were customized using Agilent eArray 6.0, according to the manufacturer's recommendations (https://earray.chem.agilent.com/earray/). Each customized microarray (8 × 15 K) contained portions identical with 7198 gene-specific 60-mer oligonucleotide probes interrogating the 7198 predicted ORFs in *S. erythraea* (as reported for the *S. erythraea* genome at http://131.111.43.95/gnmweb/index.html). RNA was extracted from samples derived from two independent culture samples collected at two time points (24 and 48 h) using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA quality and quantity were determined by nanodrop UV spectroscopy (Ocean Optics) and analysis on an RNA 6000 Nano LabChip (Agilent Technologies) using a 2100 bioanalyzer (Agilent Technologies). RNA samples were processed and hybridized to the customized chips SER v1.0. Microarray data was normalized by the Agilent Feature Extraction software (Agilent Technologies) using total array signals and LOWESS algorithm options. The gene expression ratio (*n*-fold change; Δ*dasR* strain versus WT) was calculated from the normalized signal intensities. Genes selected for heat map production with expression ratio and detailed annotation in this study are shown in Table S1. The raw DNA microarray data set analyzed in the present work has been submitted to the NCBI Gene Expression Omnibus under the accession number GSE69427.

Quantitative real-time reverse transcription PCR

Total RNA was extracted and purified from the collected cell samples using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA integrality was analyzed by 1 % agarose gel electrophoresis. About 1 μg of total RNA was reverse transcribed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). For real-time reverse transcription PCR, the SYBR premix Ex Taq™ GC Kit (Perfect Real Time, Takara) was used, and about 100 ng cDNA was added in 20 μL volume of PCR reaction. PCR reactions were performed with primers listed in Table 2. The PCR was conducted using the CFX96 Real-Time System (Bio-Rad, USA) at 95 °C for 5 min, then 40 cycles each of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 30 s and finally followed by an extension at 72 °C for 10 min.

Scanning electron microscopy

S. erythraea strains were cultivated on R2YE agar plates, covered with plastic cellophane, at 30 °C. At the specified time, a piece of cellophane covered with mycelia was extracted and fixed with 2 % osmium tetroxide for 24 h and then dehydrated by air drying for 1 h. Each specimen was sputter coated with platinum-gold and examined with a Hitachi S4000 scanning electron microscope.

Results

Deletion of *dasR* leads to decreased erythromycin production in *S. erythraea*

In order to assess the regulatory role of DasR in antibiotic production, a *dasR* in-frame deletion mutant strain was previously constructed by replacing the 756 nt of the SACE_0500 gene (GenBank accession no. NC_009142.1 553254–554009) by the thiostreptone resistance cassette (Liao et al. 2014a). The *S. erythraea* wild-type (WT) and *dasR* null mutant (Δ*dasR*) strains were cultivated on R2YE agar plates covered with plastic cellophane, and the growth biomass and antibiotic production were measured throughout the growth course. We found that the two strains showed a slight discrepancy in terms of growth rate and biomass accumulation (Fig. 1a). Notably, the deletion of *dasR* led to an obvious reduction of erythromycin production throughout the growth stage (Fig. 1b), indicating that DasR plays a positive role in antibiotic biosynthesis in *S. erythraea*.

To elucidate the molecular mechanism underlying the function of DasR in antibiotic production, a comparative transcriptome analysis was performed using a genome-wide DNA microarray to investigate the gene expression profiles of *S. erythraea* WT and Δ*dasR* strains. Each strain was grown in liquid TSB medium and total RNA were extracted from the mycelia sample harvested at 24 and 48 h, respectively. We tested the expression profiles of the *ery* cluster, which was

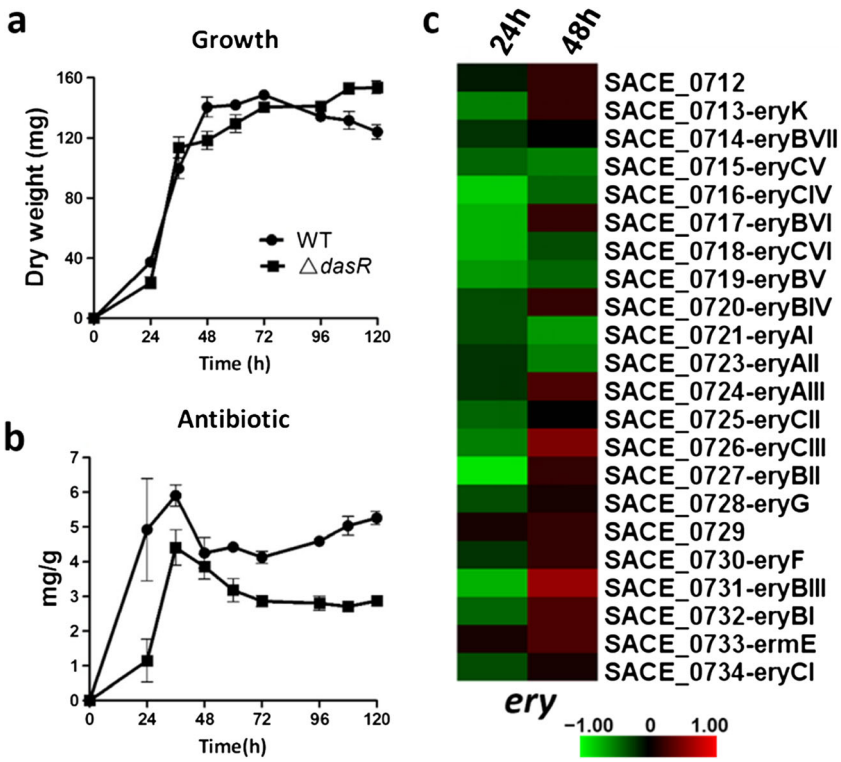

Fig. 1 Phenotype of *dasR* null mutant (Δ*dasR*) compared with that of the wild-type strain (WT) of *S. erythraea*. **a** Growth curves of WT and Δ*dasR* strains on R2YE agar. **b** Antibiotic production of WT and Δ*dasR* strains on R2YE agar. **c** Transcription profiles of the erythromycin (*ery*) biosynthesis cluster. WT and Δ*dasR* were grown in TSB medium, and RNA samples were extracted at 24 and 48 h. The relative changes of gene transcription are determined using $\log_2$ (ratio of Δ*dasR*/WT) and shown on a color scale, with *red* representing an increase in transcript abundance and *green* indicating a decrease in the *dasR* mutant, relative to wild-type. *Black* represents unchanged expression levels. *Error bars* indicate standard deviations from three independent biological replicates

directly related to erythromycin biosynthesis (Fig. 1c); however, no obvious correlation was revealed between the expression of the *ery* cluster and the decreased erythromycin phenotype in Δ*dasR*. Most of the *ery* genes were shown to be downregulated at the early growth stage (24 h), whereas about half of the genes were upregulated at the stationary growth stage (48 h), which was inconsistent with the results of the phenotypic study (Fig. 1b). It should be noted that the fold change of the *ery* cluster between WT and Δ*dasR* was slight (<2-fold change). This result suggests that the regulatory effects of DasR on antibiotic production are not produced by a direct impact on erythromycin biosynthetic genes.

DasR has a major impact on the feeder pathway of erythromycin biosynthesis

Given the significant role of the feeder pathway in affecting the erythromycin biosynthesis, we checked the transcription profiles of related genes, as shown in Fig. 2a. Comparative transcriptome analysis showed obvious downregulated fold change expressions of the majority of these genes, with the exception of SACE_3241, SACE_3399, and SACE_4237, which were upregulated at 24 or 48 h (Fig. 2b). Additionally, to validate the microarray analysis, the transcription of SACE_5368-5340, SACE_6238, and SACE_1456-1459 were measured by quantitative real-time reverse transcription PCR (qRT-PCR) using cDNA templates generated from an individually cultivated sample collected at the stationary stage (48 h) (Fig. 2c, d). qRT-PCR analysis showed high consistency with the microarray data in the trends of expression changes, as SACE_1456-1459 was decreased at approximately 50 % of the transcription, and SACE_5368-5340 was decreased at approximately 30~40 % of the transcription in Δ*dasR*, compared with WT, at 48 h. However, no difference in the expression of SACE_6238 was observed in the two strains. Taken together, these results suggest that the decreased productivity of erythromycin in the absence of *dasR* was due to the downregulation of most of the genes involved in the major feeder pathway.

DasR activates the pigment biosynthesis via direct regulation of the *rpp* cluster

S. erythraea produce a soluble, diffusible reddish pigment, the product of chalcone synthase encoded by the red-brown pigment producing (*rpp*) locus that belongs to type III polyketide synthase (PKS) (Cortes et al. 2002). Pigment itself is also an important secondary metabolite whose generation is a visible indicator for onset of secondary metabolism. Microarray analysis showed that the transcriptional levels of *rpp* cluster

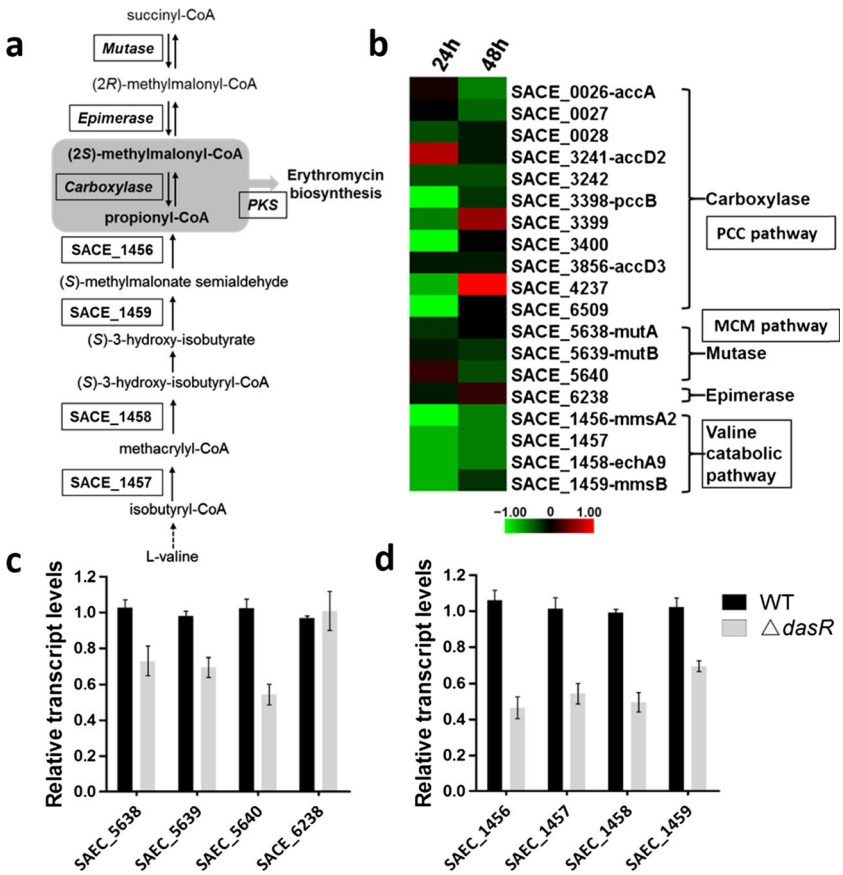

Fig. 2 Transcriptome analysis of the feeder pathway of erythromycin biosynthesis. **a** Putative metabolic pathways leading to the production of propionyl-CoA and 2-methylmalonyl-CoA, two precursors for the biosynthesis of the erythromycin precursor, 6-deoxyerythronolide B. **b** Heatmap of the gene clusters involved in the erythromycin feeder pathway. The relative changes of gene transcription are determined using $\log_2$ (ratio of Δ*dasR*/WT) and shown on a color scale. *Red* = upregulation; *green* = downregulation. **c**, **d** qRT-PCR analysis of the transcripts of the feeder pathway genes, at 48 h. Relative transcript levels were obtained individually after normalization to the internal reference gene 16S rRNA. Gene expression values observed in WT were set to 1.0. *Error bars* indicate standard deviations from three independent biological replicates

(SACE_1241-1243) were no obvious change at 24 h whereas decreased significantly at 48 h in Δ*dasR* compared to WT (Fig. 3a). Notably, a putative *dre* site (ACGGGATGACCA) was found in upstream of *rpp* locus (Fig. 3b), which was highly similar to the previous identified consensus (TGGTCTAGACCA) of *S. erythraea* and *S. coelicolor*. To determine the possible interaction of DasR with the promoter regions of *rppA*, electrophoretic mobility shift assays (EMSAs) were conducted using purified His-tagged DasR proteins with biotin-labeled DNA probes harboring the potential *dre* sequence. EMSA demonstrated His-tagged DasR bind directly to the *rppA* promoter region (Fig. 3c). We subsequently performed qRT-PCR to investigate the regulatory impact of *dasR* deletion to the expression of *rpp* locus. Transcription of *rpp* locus decreased 50 % in Δ*dasR* compared to WT (Fig. 3d) which was well in accordance with the transcriptome data (Fig. 3a). Consequently, the pigment production was expectedly reduced in Δ*dasR* grown on R2YE agar media (Fig. 3e, f). These results demonstrated that DasR was a crucial regulator which is essential for pigment biosynthesis via directly control the *rpp* locus in *S. erythraea*.

Disruption of *dasR* causes delayed morphological defect

In *S. coelicolor*, the deletion of *dasR* caused a non-sporulating phenotype in the mutant strain, indicating that *dasR* is a member of the *bld* genes, which are known to play important roles in development (Rigali et al. 2006). We investigated whether DasR played a similar role in the control of morphological development in *S. erythraea*. The *S. erythraea* WT and Δ*dasR* strains were streaked on a R2YE agar plate, supplemented with 0.5 % glucose as the sole source of carbon. It was observed that the disruption of *dasR* caused delayed morphological development, compared with the wild-type strain, at the early growth stage (<3 days) (Fig. 4a). Scanning electron microscopy (SEM) further revealed that the WT strain presented fuzzy hyphae, while Δ*dasR* presented smooth hyphae (Fig. 4b), corresponding with the degree of morphogenesis observed in Fig. 4a. However, Δ*dasR* did not lose the capability of producing aerial hyphae, as prolonged incubation (>3 days) lead to normal development (data not shown). This suggests that the *dasR* homologue of *S. erythraea* is not a strict *bld* regulatory gene, as described in *S. coelicolor*, whereas it indeed affected development only at the early growth stage. We then analyzed the expression profiles of the genes related to morphogenesis, namely the six *whi* family regulatory genes identified in *S. erythraea* (Oliynyk et al. 2007) (Fig. 4c). However, the intricate expression profiles of these *whi* genes showed no consistency with the developmental delay. qRT-PCR was conducted which further showed that transcription of *whiB3* (SACE_5583) and *whiB2* (SACE_6464) were notably downregulated, whereas others with no obvious difference or upregulated at 48 h (Fig. 4d). We also found that *bldD*, a key developmental regulator gene required for erythromycin biosynthesis and morphological differentiation (Chng et al. 2008), was significantly downregulated to about 70 % transcription in Δ*dasR*, as observed from the microarray and qRT-PCR data (Fig. 4c, d). Therefore, the delayed morphological development phenomenon observed in Δ*dasR* might be associated with the reduced transcription of *bldD* and part of *whi* family.

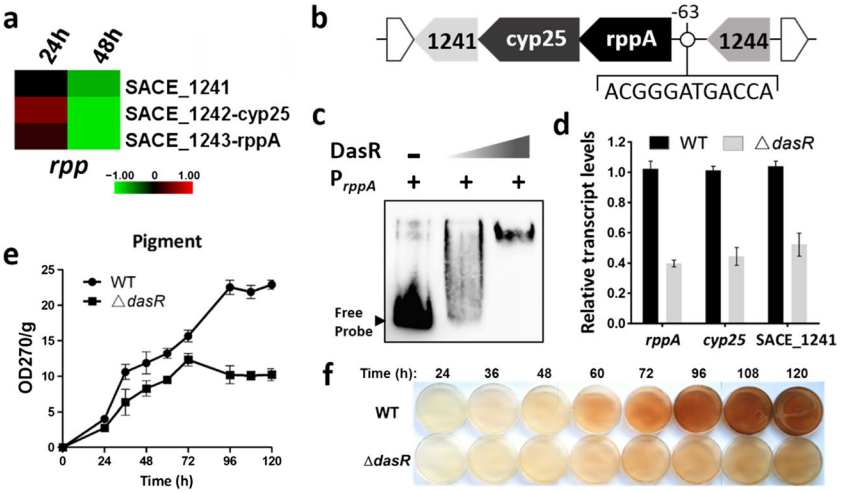

Fig. 3 DasR directly regulates pigment biosynthesis in *S. erythraea*. **a** Heatmap of the *rppA* gene cluster. The relative changes of gene transcription are determined using $\log_2$ (ratio of Δ*dasR*/WT) and shown on a color scale. *Red* = upregulation; *green* = downregulation. **b** Putative *dre* site within the upstream region of *rpp* locus. **c** EMSAs with pure His-tagged DasR and DNA probe harboring *dre* site of *rppA*. An excess of poly(d[I-C]) was included in every lane as an internal control to avoid unspecific binding of the protein to the DNA. **d** qRT-PCR analysis of the transcription profiles of the *rpp* cluster at 48 h. Relative transcript levels were obtained individually after normalization to the internal reference gene 16S rRNA. **e**, **f** Pigment biosynthesis of WT and Δ*dasR* strains in R2YE agar. Pictures were representatives from three independent biological replicates. *Error bars* indicate standard deviations from three independent biological replicates

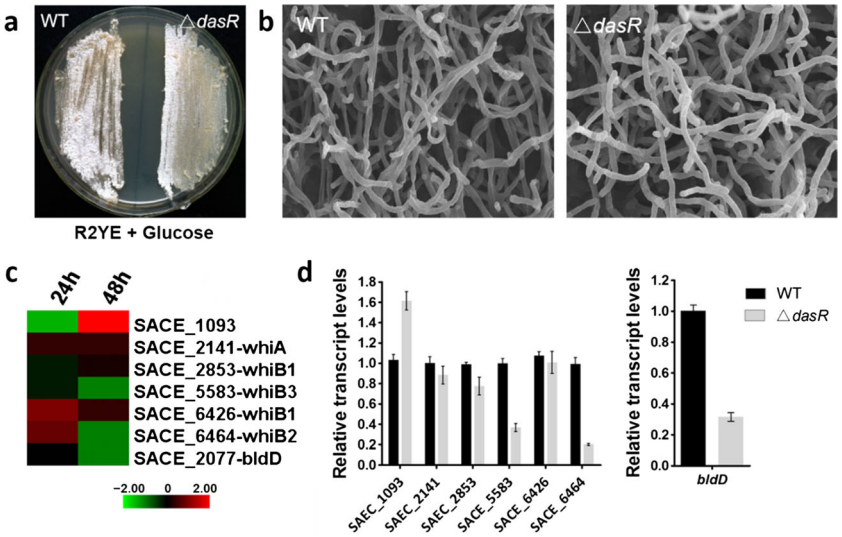

Fig. 4 Disruption of *dasR* causes delayed development, compared with that of the wild-type strain. **a** Growth behavior of WT and Δ*dasR* on R2YE agar supplemented with 0.5 % glucose. **b** Scanning electron micrographs of WT and Δ*dasR* grown on R2YE agar supplemented with 0.5 % glucose. The pictures are shown at ×3000 magnification. **c** Transcription profiles of the regulatory genes related to morphogenesis. The relative changes of gene transcription are determined using $\log_2$ (ratio of Δ*dasR*/WT) and shown on a color scale. *Red* = upregulation; *green* = downregulation. **d** qRT-PCR analysis of the transcription profiles of *whi* family and *bldD* genes at 48 h. *Error bars* indicate standard deviations from three independent biological replicates

The intricate effects of GlcNAc on secondary metabolism and morphogenesis

Recently, the pleiotropic effects of *N*-acetylglucosamine (GlcNAc) on development and antibiotic biosynthesis have been reported in *S. coelicolor*. It has been shown to regulate development and antibiotic production conditionally, namely by blocking these processes under GlcNAc-rich conditions, whereas stimulating them under GlcNAc-poor conditions. In addition, the assimilation of GlcNAc leads to an accumulation of GlcN-6-P, which is the allosteric effector, and blocks the regulatory activity of DasR, indicating a direct linkage between the regulatory effects caused by GlcNAc and DasR.

To assess the effect of GlcNAc on secondary metabolism in *S. erythraea*, we streaked the wild-type strain on R2YE agar plates supplemented with glucose, GlcNAc, maltose, mannitol, fructose, or glycerol as the sole carbon source, respectively. After 6 days of incubation, it was shown that GlcNAc clearly hindered development and pigment production in comparison with the other carbon sources (Fig. 5a). Additionally, GlcNAc also negatively affected the antibiotic biosynthesis of *S. erythraea* in liquid TSB media (Fig. 5b). Furthermore, to demonstrate the negative regulatory effects of GlcNAc associated with DasR regulation, WT and Δ*dasR* strains were grown on R2YE supplemented with GlcNAc as the sole source of carbon, and the growth status was tested at

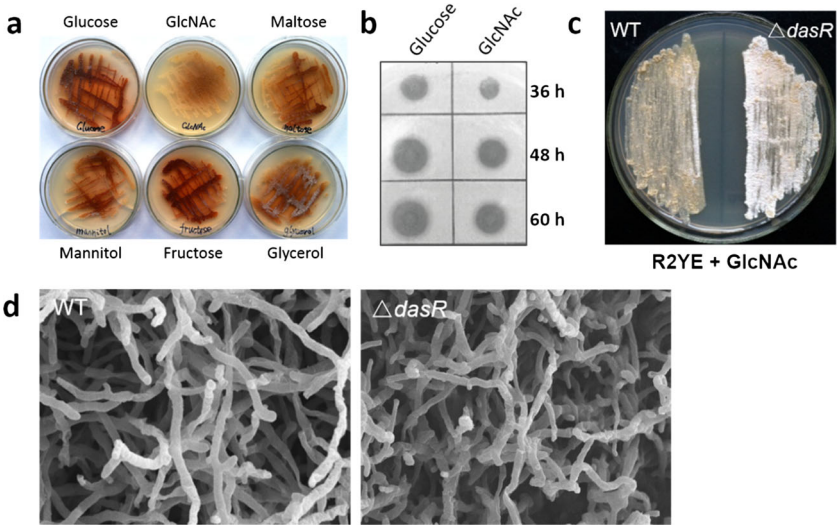

Fig. 5 Effects of GlcNAc on development and secondary metabolite production. **a** Phenotype of *S. erythraea* grown on R2YE containing 0.5 % (*w*/*v*) of various carbon sources. **b** GlcNAc blocks antibiotic production in TSB medium supplemented with 0.5 % glucose or GlcNAc as the sole source of carbon. **c** Growth behavior of WT and Δ*dasR* on R2YE supplemented with 0.5 % GlcNAc. **d** SEM of WT and Δ*dasR* grown on R2YE supplemented with 0.5 % GlcNAc. The pictures are shown at ×3000 magnification

3 days by direct observation (Fig. 5c) or by SEM (Fig. 5d). Development of the WT strain was notably inhibited, as observed in Fig. 5a. Interestingly, Δ*dasR* undertook the normal morphogenetic program, which was further confirmed by SEM (Fig. 5d), suggesting that the effects of GlcNAc were disabled in the absence of *dasR*. Collectively, these results suggested that GlcNAc played a negative role in the development and secondary metabolite production of *S. erythraea* and that this regulatory effect was in relation with DasR.

Discussion

The inactivation of *dasR* is shown to affect secondary metabolism and sporulation in *Streptomyces* (Rigali et al. 2006; Rigali et al. 2008); in fact, *das* stands for "deficient in aerial hyphae and spore formation" (Seo et al. 2002), which shows that the significant role of DasR in development was understood through phenotypic studies before the discovery of its physiological role in the control of GlcNAc and chitin metabolism. Subsequently, the regulatory function of DasR in primary metabolism was also characterized in *S. erythraea* (Liao et al. 2014a). The comprehensive regulon identification suggested that DasR stands in the hub of interplay between primary and secondary metabolism. In this study, we have demonstrated through developmental studies and transcriptome/transcription analysis that DasR is a pleiotropic regulator that is required for morphogenesis and antibiotic/pigment production in *S. erythraea*.

With increased understanding of the regulatory role of DasR in different species, however, remarkable discrepancies in its underlying mechanisms have been found between *S. erythraea* and *S. coelicolor*. One major difference is that the *ery* cluster lacks CSR genes, which means that DasR regulates erythromycin biosynthesis by an unknown mechanism in *S. erythraea*. Here, we found that DasR neither bound directly to any promoter region of the *ery* cluster (data not shown) nor significantly affected its expression. Notably, DasR plays a major role in the feeder pathway of erythromycin biosynthesis, which would be a probable explanation of the decreased erythromycin production in Δ*dasR*. Furthermore, the downregulation of *bldD* in Δ*dasR* suggests an indirect erythromycin control pattern of DasR. Another difference between *S. coelicolor* and *S. erythraea* is that compared to the indirect control of antibiotic biosynthesis clusters by repressing the CSR genes, DasR could directly control the pigment biosynthetic *rpp* cluster as well as the *PKS*3 cluster (Oliynyk et al. 2007) (data not shown). These findings highlight DasR as a novel regulator that may directly regulate the secondary metabolite genes in *S. erythraea*.

In addition, DasR may also play a role in the morphological development of *S. erythraea*. DasR was shown to be essential for development, as the deletion of *dasR* resulted in a bald phenotype, as suggested by the studies of *S. coelicolor* (Rigali et al. 2006) and *Streptomyces griseus* (Seo et al. 2002). However, it is interesting to note that the inactivation of *dasR* inhibited the formation of aerial hyphae only at the early growth stage, while Δ*dasR* produced aerial hyphae normally with a prolonged growth period. One plausible hypothesis would be that a secondary, DasR-like regulatory pathway may be present in *S. erythraea*. It should also be noted that another GntR/HutC family regulator, designated as DasR2 (SACE_2060), a possible alternative of DasR, was presented in *S. erythraea*. Bioinformatic analysis showed that the overall amino acid conservation between DasR and DasR2 was low; however, the DNA-binding domains were highly conserved, as six out of the seven residues predicted to be involved in DNA binding were conserved between DasR and DasR2 (Liao et al. 2014a). In addition, DasR2 was able to bind a majority of the *dre* sequences recognized by DasR (unpublished data). Therefore, DasR2 may possibly take over the role of DasR in the regulation of the regulon, including those affecting development, in the absence of *dasR*. A competitive regulatory mechanism between DasR and DasR2 in regards to the control of the genes involved in secondary metabolism is still under investigation.

We also discussed the GlcNAc signal and its response and regulation mediated via DasR. GlcNAc supply was previously shown to either trigger or inhibit development and secondary metabolite production in *Streptomyces*, depending on the culture conditions. In the current study, we mainly used nutrient-rich conditions (R2YE agar or TSB liquid) for a practical concern. High levels of GlcNAc were shown to repress the development and erythromycin production of *S. erythraea*. Remarkably, the presence of GlcNAc abolished the regulation of DasR, as the growth of WT in the presence of GlcNAc exhibited a Δ*dasR*-like phenotype in R2YE agar supplemented with glucose. Additionally, GlcN-6-P, the catabolic intermediate of the GlcNAc pathway, can modify the DNA-binding activity of DasR, as demonstrated previously (Liao et al. 2014a; Rigali et al. 2006). Therefore, these results suggest that high concentrations of GlcNAc mimic the *dasR* null effect by inhibiting the development of wild-type *S. erythraea*. On the other hand, Δ*dasR* exhibited normal morphological program compared to the bald phenotype of WT, in the presence of GlcNAc, suggesting that the effects of GlcNAc are unquestionably mediated by DasR. In general, our study elucidated the regulatory role of DasR in the development and secondary metabolite production, in combination with its response to GlcNAc signal, in *S. erythraea*. These results could provide promising biotechnological information, which may be used for the genetic manipulation of *S. erythraea* to improve the production of secondary metabolites.

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Conflict of interest The authors declare that they have no competing interests.

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