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# A novel function of *Streptomyces* integration host factor (sIHF) in the control of antibiotic production and sporulation in *Streptomyces coelicolor*

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**Abstract** Bacterial integration host factors (IHF) play important roles in site-specific recombination, DNA replication, transcription, genome organization and bacterial pathogenesis. In *Streptomyces coelicolor*, there are three putative IHFs: SCO1480, SCO2950 and SCO5556. SCO1480 or *Streptomyces* IHF (sIHF) was previously identified as a transcription factor that binds to the promoter region of *redD*, the pathway-specific regulatory gene for the undecylprodigiosin biosynthetic gene cluster. Here we show that production of the pigmented antibiotics actinorhodin

and undecylprodigiosin is strongly enhanced in *sihf* null mutants, while sporulation was strongly inhibited, with an on average 25% increase in spore size. Furthermore, the *sihf* mutant spores showed strongly reduced viability, with high sensitivity to heat and live/dead staining revealing a high proportion of empty spores, while enhanced expression of sIHF increased viability. This suggests a major role for sIHF in controlling viability, perhaps via the control of DNA replication and/or segregation. Proteomic analysis of the *sihf* null mutant identified several differentially expressed transcriptional regulators, indicating that sIHF may have an extensive response regulon. These data surprisingly reveal that a basic architectural element conserved in many actinobacteria such as mycobacteria, corynebacteria, streptomycetes and rhodococci may act as a global regulator of secondary metabolism and cell development.

Yung-Hun Yang and Eunjung Song have contributed equally to this work.

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## Introduction

Bacterial integration host factors (IHF) play important roles in site-specific recombination, DNA replication, transcription, genome organization and bacterial pathogenesis (Pedulla et al. 1996; Mangan et al. 2006). IHF binds a conserved DNA sequence, and changes the conformation by locally bending the

DNA (Rice 1997). IHF is known to promote long-range interactions in several systems (Goosen and van de Putte 1995) and affects local DNA conformational structures critical to transcription control.

A new type of IHF was first identified and studied in mycobacteria, which is essential for their survival, but shares relatively low amino acid identity to the canonical IHF (Pedulla and Hatfull 1998; Pedulla et al. 1996). Mycobacterial IHF (mIHF) has several orthologs in actinobacteria, including corynebacteria, streptomycetes and rhodococci, and we propose to refer to the family as actinobacterial IHF (aIHF). mIHF itself is an unusual host factor and is required for the formation of recombinogenic intasomes, which are nucleoprotein complexes active in the integration of bacteriophage DNA into host DNA (Pedulla and Hatfull 1998). However, mIHF does not specifically bind to the site of integration (*L5 attP*). The different types of IHFs act as architectural elements that control transcription by facilitating the formation of DNA–protein complexes, and/or function as direct transcriptional regulators by themselves without involvement of any other transcription factors (Goosen and van de Putte 1995).

In *Streptomyces coelicolor*, a model antibiotic producer (Bentley et al. 2002; Bibb 2005; van Wezel and McDowall 2011), there are three putative IHFs: SCO1480, SCO2950 and SCO5556 (<http://strepdb.streptomyces.org.uk/> (Streptomyces database website)). Recently, SCO2950 was revealed as the conventional nucleoid-associated HU protein, HupA that is similar to *Escherichia coli* HU $\alpha$  and HU $\beta$  (Salerno et al. 2009). It also shows high homology with known IHFs such as HimA (Ihf $\alpha$ ) and HimD (Ihf $\beta$ ) from *E. coli* (Supplementary Fig. 1). Another HU-family protein, SCO5556, contains N-terminal part similar to HU proteins and a C-terminal domain similar to the alanine- and lysine-rich C termini of eukaryotic linker histones. Such two-domain HU proteins were found to exist only in actinobacteria, and designated as HupS (Salerno et al. 2009).

It is known that the IHF is structurally and functionally similar to histone-like protein HU in *E. coli* (Takeuchi et al. 2002). HU often compensates for the absence of IHF in some cellular functions such as cell growth and replication of *oriC* plasmids (Kano et al. 1991), but IHF cannot compensate for the absence of HU in the cell, even though the IHF and HU share over 30% of amino acid identity (Boubrik et al.

1991). IHFs in *E. coli* have pleiotropic functions, and besides being involved in crucial cellular functions, they have also been reported to directly control the transcription of at least 59 operons (Karp et al. 2002).

We previously reported that SCO1480, which is a member of the aIHF family with high sequence homology to mIHF, binds to the promoter regions of *actII-ORF4* and *redD*, which encode the pathway-specific activators for actinorhodin (ACT) and undecylprodigiosin (RED) biosynthesis, respectively (Park et al. 2009). Unlike other well-known IHF homologs, the sequence of SCO1480 (*Streptomyces* IHF, sIHF) is conserved only in actinobacteria such as mycobacteria, corynebacteria, streptomycetes, and rhodococci, but its function has been only reported in mycobacteria (Pedulla and Hatfull 1998). To obtain more detailed insight into the transcriptional regulatory role of *Streptomyces* IHF (further referred to as sIHF), we scrutinized the *sihf* (SCO1480) null mutant in terms of growth, antibiotic production and development. This is the first study of a different role of an aIHF, known as one of basic architectural units (Charlier et al. 1995; Devroede et al. 2006), on secondary metabolisms in *Streptomyces*.

## Materials and methods

### Bacterial strains and media composition

All of the *E. coli* and *Streptomyces* strains used in this experiment are listed in Table 1. Cultivation of *S. coelicolor* strains followed the standard procedures (Kieser et al. 2000). Briefly, fresh M145 spores were collected on R5<sup>−</sup> media composed of 103 g sucrose, 0.25 g K<sub>2</sub>SO<sub>4</sub>, 10.12 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 g glucose, 0.1 g Difco casamino acids, 5 g yeast extract, 5.73 g TES buffer, 7 ml of 1 N NaOH, and 2 ml of a trace element solution in 1 l of distilled water. Minimal media with different amino acids and sugars contained 0.5 g amino acid, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, and 10 g sugar as carbon source with 0.01 g FeSO<sub>4</sub>·7H<sub>2</sub>O in 1 l of distilled water. Mannitol soya flour medium (SFM) is composed of 20 g agar, 20 g of mannitol, 8 g of soya flour, made up to 1 l with tap water.

### Protein over-expression in *E. coli*

For the expression of SCO1480 with a C-terminal 6× His-tag, the coding region was amplified by PCR

**Table 1** Bacterial strains, plasmids, and primers used in this study

| Strains, plasmids, and primers | Relevant information                                                                                                                                                                                                       | Source or reference |
|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| <b>Bacterial strains</b>       |                                                                                                                                                                                                                            |                     |
| <i>E. coli</i> strains         |                                                                                                                                                                                                                            |                     |
| DH5 $\alpha$                   | F <sup>−</sup> $\phi$ 80 <i>lacZ</i> M15 <i>endA</i> <i>recA</i> <i>hsdR</i> (r <sub>k</sub> <sup>−</sup> m <sub>k</sub> <sup>−</sup> ) <i>supE</i> <i>thi</i> <i>gyrA</i> <i>relA</i> $\Delta$ ( <i>lacZYA-argF</i> )U169 | Laboratory stock    |
| BL21(DE3)                      | F <sup>−</sup> <i>ompT</i> <i>hsdS<sub>B</sub></i> (r <sub>B</sub> <sup>−</sup> m <sub>B</sub> <sup>−</sup> ) <i>gal</i> <i>dcm</i>                                                                                        | Novagen             |
| JM110                          | <i>dam</i> <sup>−</sup> , <i>dcm</i> <sup>−</sup>                                                                                                                                                                          | Laboratory stock    |
| BW25113                        | K12 derivative: $\Delta$ <i>araBAD</i> , $\Delta$ <i>rhaBAD</i>                                                                                                                                                            | Gust et al. (2003)  |
| <i>S. coelicolor</i>           |                                                                                                                                                                                                                            |                     |
| A3(2) M145                     | SCP1 <sup>−</sup> , SCP2 <sup>−</sup> , Pgl <sup>+</sup>                                                                                                                                                                   | KCTC                |
| BG716                          | SCO1480 deleted mutant                                                                                                                                                                                                     | This study          |
| BG7161                         | BG716 carrying pYH81                                                                                                                                                                                                       | This study          |
| BG7164                         | M145 carrying pYH81                                                                                                                                                                                                        | This study          |
| BG7165                         | BG716 carrying pEJ70                                                                                                                                                                                                       | This study          |
| <b>Plasmids</b>                |                                                                                                                                                                                                                            |                     |
| pET24ma                        | p15A replication origin, T7 lac promoter, C-terminal his-tag coding, kan <sup>R</sup>                                                                                                                                      | Yang et al. (2005)  |
| pIBR25                         | pWHM3 carrying ermE* promoter ( <i>EcoRI/KpnI</i> ) from <i>Saccharopolyspora erythraea</i>                                                                                                                                | Thuy et al. (2005)  |
| pSET152-neo                    | Apramycin resistance gene-deleted pSET152 ( <i>Streptomyces</i> integration vector) harboring neomycin resistance gene                                                                                                     | This study          |
| pYH80                          | pET24ma carrying PCR product of SCO1480 from <i>S. coelicolor</i>                                                                                                                                                          | This study          |
| pYH81                          | pIBR25 carrying PCR product of SCO1480 from <i>S. coelicolor</i>                                                                                                                                                           | This study          |
| pEJ70                          | pSET152-neo carrying PCR product of SCO1480 together with 223 bp upstream of SCO1480 as promoter                                                                                                                           | This study          |
| pIJ773                         | <i>aac(3)IV(apra<sup>R</sup>) + oriT</i>                                                                                                                                                                                   | Gust et al. (2003)  |
| <b>Primers</b>                 |                                                                                                                                                                                                                            |                     |
| 1480-E. coli                   | CGTGGATCCGTGGCTCTTCCGCCCTTAC/ACGAAGCTTTCAGTGGT<br>GGTGGTGGTGGTGGCTGCCGGTGCTGCCGA                                                                                                                                           |                     |
| 1480-St                        | CGTCGTGGATCCGTCTCGGGCGAGCGTGTTGC/CGTCGTAAGCTT<br>TCAGCTGCCGGTGCTGCCGAA                                                                                                                                                     |                     |
| 1480+prom-St                   | ATAATAT GCGGCCG CCCTGGATACATCCTCAAATC/ATAAT TCTAGA<br>GACTCAGCTGCCGGTGCTGC                                                                                                                                                 |                     |
| Del-1480F                      | CGTATCCGACAGTTCGACATCCGAGGTGACGTAGGC GTGATTCCGGG<br>GATCCGTCGACC                                                                                                                                                           |                     |
| Del-1480B                      | CAGCGATTATTCCAGCAATCCCGGAGTGGTCCGGAC TCATGTAGGCTG<br>GAGCTGCTTC                                                                                                                                                            |                     |
| Del-confirm                    | CTGACCAGGACTTTTCCGCTG/GAGACCGACAGCCATACCTCG                                                                                                                                                                                |                     |
| Neomycin                       | ATATAT GAGCTC ATGATTGAA CAAGATGGAT/ATATAT GAGCTC TCA<br>GAAGAACTCGTCAAGAAG                                                                                                                                                 |                     |
| EMSA-redZ                      | CCGGCGCGCGGACGCCGCGTGCA/ACGTTGACTTTCGTCCAGGAAGGA                                                                                                                                                                           |                     |
| EMSA-sihf                      | GTCCCCGACGTGACCGCGTTGCGGA/GCCTACGTACCTCGGATGTCGAA                                                                                                                                                                          |                     |
| EMSA-pyrRbldD                  | CATATGGCTCCCCGGACAAGGTGT/ACGAAACGGACCCCTTCTCCGCC                                                                                                                                                                           |                     |

using *S. coelicolor* chromosomal DNA as a template and the primers listed in Table 1. The amplified fragments were digested with *Bam*HI plus *Hind*III and cloned into pET24ma. After no errors in PCR were confirmed by nucleotide sequencing, the constructed plasmids were transformed into *E. coli* BL21. Cells harboring each plasmid were grown at 37°C in 50 ml LB broth with 50 µg/ml kanamycin. When the culture reached an absorbance of approximately A<sub>600</sub> of 0.6, protein expression was induced with 0.1 mM isopropyl thio-β-D-galactopyranoside. After 6 h at 30°C, cells were washed twice with 20 ml ice-cold phosphate buffered saline (PBS) and resuspended with 5 ml Buffer I (50 mM phosphate buffer pH 8.0, 0.01% Tween 20, and 100 mM NaCl supplemented with 20 mM imidazole) and then sonicated for 20 min in an ice bath. Cell debris was removed by centrifugation. The resultant supernatant was applied to Ni-NTA (Qiagen) mini-column pre-equilibrated with buffer I. His-tagged proteins were eluted with 1 ml of elution buffer (50 mM phosphate buffer pH 8.0, 0.01% Tween 20, and 100 mM NaCl supplemented with 250 mM imidazole). The purified protein concentration was measured using a Bradford assay (Bio-Rad, USA) and the samples were stored at –20°C until further use.

#### Electrophoretic mobility shift assay (EMSA)

PCR amplified DNA fragments containing the promoters of *redZ*, *sihf* and *pyrR-bldD* were prepared using the relevant primers (Table 1) and radiolabeled with T4 polynucleotide kinase in the presence of [ $\gamma$ -<sup>32</sup>P]-dATP, and the unreacted [ $\gamma$ -<sup>32</sup>P]-dATP was removed using ProbeQuant™ G-50 Micro Columns (GE Healthcare, USA). The labeled probes were incubated with His-tag purified siHF at 37°C for 10 min followed by an ice bath for 30 min. The total volume containing 20 mM HEPES (pH 7.8), 10% w/v glycerol, 100 mM KCl, 0.05 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.01% Nonidet P-40, and 2 µg sheared salmon sperm DNA (ssDNA) was 20 µl. Protein-bound DNA and free DNA were resolved on 5% acrylamide gel in 0.5× TBE buffer at room temperature. The gel was exposed overnight. Autoradiography was performed overnight and analyzed with a Typhoon 8600 scanner (GE Healthcare, USA).

#### Construction of deletion mutants

Using the PCR-targeted Redirect method (Gust et al. 2003), the complete coding region of SCO1480 (*sihf*) located on *S. coelicolor* cosmid clone St9C5 (obtained from the John Innes Centre, Norwich, UK) was replaced by the apramycin resistance gene *aac(3)IV*. For this construction, oligonucleotide primers (Table 1) were designed such that one contained the –36/+3 region relative to the start of SCO1480 as well as the upstream pIJ773 priming site, and the second the stop codon and 36 nt downstream region of SCO1480 as well as the downstream pIJ773 priming site; following PCR amplification using pIJ773 as the template, the DNA fragment was used to generate a knock-out cosmid based on the Redirect technology (Gust et al. 2003), which was then used for the replacement of SCO1480 by the apramycin resistance cassette *aacC4* (Table 1). For this, the mutated cosmid was introduced by conjugation into *S. coelicolor* M145 and colonies were selected that had undergone homologous recombination, with the desired double recombinants carrying apramycin resistance, while being sensitive to kanamycin, the selectable marker for the vector sequences. The deletion of SCO1480 was confirmed by sequencing the corresponding PCR products and by complementation (Table 1). The mutant was designated BG716. In this study, BG7161 and BG7165 were constructed for the complementation of SCO1480 (Table 1). In BG7161, SCO1480 is cloned in a multi-copy vector based on pIBR25, harbouring the *sihf* gene behind the *ermE*\* promoter. In BG7165, SCO1480 is cloned in the promoter-less pSET152-neo integration vector. To express *sihf* from its native promoter, the *sihf* gene with 223 bp upstream region was cloned as a *Not*I-*Xba*I fragment into pSET152-neo, a derivative of pSET152 (Bierman et al. 1992) harbouring the neomycin resistance cassette *aph*, thus generating BG7165.

#### ACT and undecylprodigiosin antibiotics measurement

Mycelia of M145 and BG716 were cultured on R5<sup>–</sup> agar plates and circular agar blocks (5 mm diameter) containing the cells were taken each day. The samples were stored at –20°C until use. The agar blocks were mashed and sonicated after treating with 1 N KOH. After the 5 min of incubation, samples were centrifuged and the A<sub>630</sub> of the supernatants was measured spectrophotometrically. The remaining cells were washed

twice with double distilled water (DDW), and the amount of RED was determined as a measure of the  $A_{530}$  following extraction with HCl-acidified methanol (pH 2). Absorbance was measured using a 96-well plate in a multiscanner (Thermo Scientific, USA). The experiments were all performed in triplicate.

### Scanning electron microscopy

The spores and hyphae of M145 and BG716 strains were observed by scanning electron microscopy (SEM) (JEOL JSM 5410LV) after 7 days of growth on minimal media containing leucine and glycerol without iron ion ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ). The cut agar blocks were pre-fixed by 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) and post-fixed by 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h, then dehydrated and dried. Each sample was sputter-coated and examined by SEM.

### Fluorescence microscopy and live/dead staining

Cell membranes were stained using a 0.2  $\mu\text{g}/\text{ml}$  solution of FM<sup>®</sup> 5-95 (*N*-(3-trimethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium-dibromide; absorbance 560 nm/emission 734 nm) prepared in Hanks' balanced salt solution. To analyse viability, the ratio of live/dead cells was determined by staining nucleic acids with a combination of the green-fluorescent SYTO<sup>®</sup> 82 stain (540/560 nm) and the red-fluorescent PI (propidium iodide; 535/617 nm) (Willemse and van Wezel 2009). All dyes were obtained from Molecular Probes, Inc (Eugene). Imaging was done as described previously (Willemse et al. 2011), using a Zeiss Axioscope A1 upright fluorescence microscope making use of a Axiocam Mrc5 camera at a resolution of 37.5 nm/pixel. The green fluorescent images were created using 470/40 nm band pass excitation and 525/50 band pass detection, for the red channel 550/25 nm band pass excitation and 605/70 band pass detection were used.

### Cell viability test

All strains were cultured in 100 ml flasks for 20 h in complex R5<sup>−</sup> liquid media, harvested and washed twice with DDW. The same amount of colony forming units (cfu) was taken for each strain and incubated in 60°C

water bath for 0, 10 or 20 min. For each time point a 10  $\mu\text{l}$  suspension was taken and serially diluted. Samples were then plated onto R5<sup>−</sup> agar plates to count the cfu.

### Two-dimensional gel analysis

WT and BG716 spread onto cellophane-covered solid minimal media containing glucose and glycine (MMGG) as a C-source and N-source, respectively, were cultured for 5 days. Then cells were harvested with spatula, resuspended and washed twice with PBS (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , and 2 mM  $\text{KH}_2\text{PO}_4$ . After the cell pellets were disrupted by sonication, the concentration of cytosolic protein was determined by a Bradford assay. Protein extracts (300  $\mu\text{g}$ ) were mixed with 350  $\mu\text{l}$  of rehydration solution and were separated by isoelectric focusing followed by polyacrylamide gel electrophoresis as described previously (Yang et al. 2010). Then gel images were analyzed by ImageMaster<sup>™</sup> 2D Platinum Software (GE Healthcare). Differentially expressed spots were tryptic digested and subjected to nLC-MS/MS (Ultimate 3000, Dionex) (LTQ orbitrap, Thermo Scientific) for peptide analysis. Proteins were identified unambiguously because two or more peptides that are detected more than three times having cross-correlation scores (Xcorr) of equal or greater than 1.7 for singly-charged, 2.5 for doubly-charged, and 3.0 for triply-charged peptides are only selected.

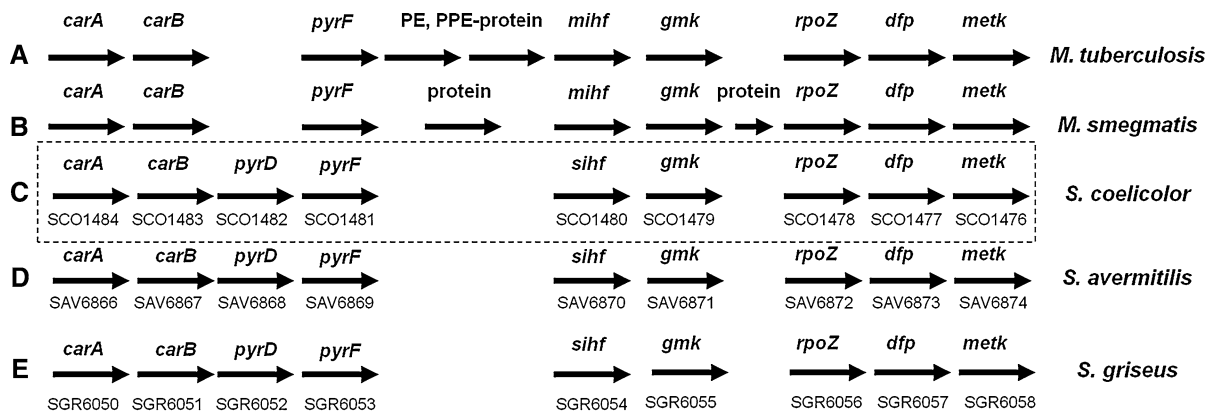
### Sequence alignment

Protein sequences of CarA, CarB, PyrD, PyrF, HU, IHF, and aIHF orthologs of mycobacteria and streptomycetes were obtained from NCBI (<http://www.ncbi.nlm.nih.gov/sites/entrez/>) (Benson et al. 1998). ClustalX 1.81 (Thompson et al. 1997) and GeneDoc2.6.002 (Nicholas et al. 2002) were used to generate and edit the multiple alignments, respectively. MEGA5.05 (Tamura et al. 2011) was used to draw the phylogenetic tree.

## Results

### Genomic environment for aIHF genes in mycobacteria and streptomycetes

The putative sIHF (SCO1480) shares relatively low homology (43% aa identity) to mIHF, the



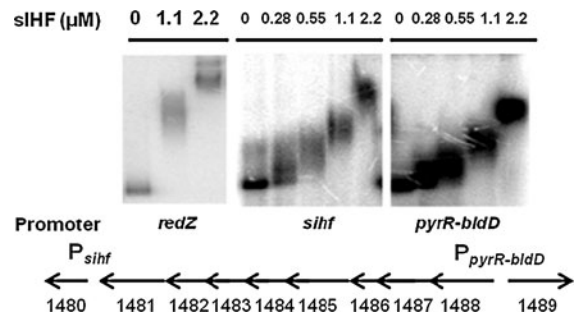
**Fig. 1** Genetic environment for the actinobacterial *ihf* gene (*aihf*) in mycobacteria (**a–b**) and streptomycetes (**c–e**) species. The gene products for the genes are as follows; *carA*: (pyrAA), carbamoyl phosphate synthase small chain, *carB*: (pyrA) carbamoyl phosphate synthase large chain, *pyrD*: dihydroorotate

dehydrogenase, *pyrF*: orotidine 5'-phosphate decarboxylase, *gmk*: guanylate kinase, *rpoZ*: omega subunit of RNA polymerase, *dfp*: DNA/pantothenate metabolism flavoprotein, *metK*: S-adenosylmethionine synthetase

IHF from *Mycobacterium smegmatis*. However, gene synteny evidence strongly suggests that SCO1480 indeed encodes sIHF, as the genetic environment of the *ihf* genes of *M. smegmatis* and *S. coelicolor* is highly similar, and both genes are located within a cluster of genes related to nucleotide biosynthesis and metabolism; these include the upstream-located *carAB* for carbamoylphosphate synthase and *pyrF* for pyrimidine synthetase, and the downstream-located *gmk* (for guanylate kinase), *rpoZ* (for the omega subunit of the RNA polymerase) and *metK* (for S-adenosylmethionine synthetase). A similar organization is found for the genes for aIHF orthologues in other actinobacteria (Fig. 1).

sIHF has a DNA binding activity that corresponds to architectural proteins

DNA affinity capture assays and EMSAs identified sIHF as a transcription factor that could bind to the promoter regions of *actII-ORF4* and *redD*, the pathway-specific activator genes for ACT and RED production, respectively (Park et al. 2009). To further analyse the role of sIHF in the control of antibiotic production, we also examined whether sIHF could bind to the promoter of *redZ*, encoding a response regulator that activates the transcription of *redD* (Guthrie et al. 1998). For this, C-terminally His<sub>6</sub>-tagged sIHF was purified following routine procedures and incubated with a <sup>32</sup>P-radiolabelled DNA probe



**Fig. 2** DNA binding studies of purified sIHF-His<sub>6</sub> with the *redZ*, *sihf* and *pyrR-bldD* promoter regions, with different amounts of purified sIHF in each lane as indicated. The relative location of the *sihf* and *pyrR-bldD* genes is indicated. For gene organization and SCO numbers see also Fig. 1

encompassing the 300 bp region upstream of the start codon of *redZ*. This indeed revealed good binding of sIHF-His<sub>6</sub> to the *redZ* promoter region (Fig. 2). Finally, sIHF also binds its own promoter and a probe corresponding to the *pyrR-bldD* intergenic region, with PyrR controlling pyrimidine biosynthesis and BldD a key regulator of development in *S. coelicolor* (Lee et al. 2007) (Fig. 2). In all cases, the probes showed stronger retardation at higher concentrations of sIHF, suggesting that multiple sIHF molecules bound to the DNA simultaneously, in line with the mode of binding for architectural proteins in mycobacteria (Pedulla and Hatfull 1998; Pedulla et al. 1996). Additionally, IHF-like proteins are known to

have low sequence specificity, which is also true for the orthologous mIHF in mycobacteria (Pedulla et al. 1996). Indeed, sIHF-His<sub>6</sub> was also shown to bind the upstream regions of genes such as *scbR* and *rok7B7* (SCO6008) and was inhibited by higher concentrations of salmon sperm DNA. Thus, sIHF has a similar mode of DNA binding as other IHF orthologues, including mIHF.

### Deletion of the gene for sIHF enhances antibiotic production

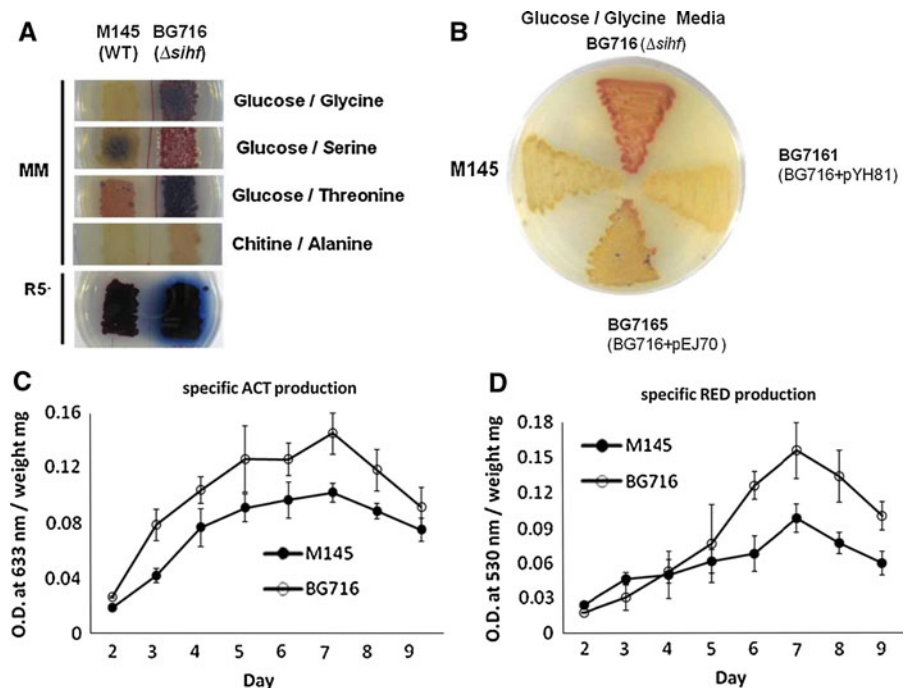
To investigate the role of sIHF in the control of growth and development, a SCO1480 null mutant was created (BG716) using PCR-targeted gene replacement. While *mIHF* is essential in *M. tuberculosis* (Cole et al. 1998), null mutants for *sihf* could be readily obtained in *S. coelicolor*. When grown under various conditions and on different carbon sources, BG716 showed major changes in production of pigmented antibiotics in comparison to the parental strain M145, with enhanced antibiotic production in minimal media as well as in R5<sup>−</sup> complex media (Fig. 3a). To confirm that the enhanced antibiotic production was solely due to the deletion of *sihf*, a complementation experiment was performed by introducing plasmid pEJ70 harbouring a wild-type copy of SCO1480 into the *sihf* null

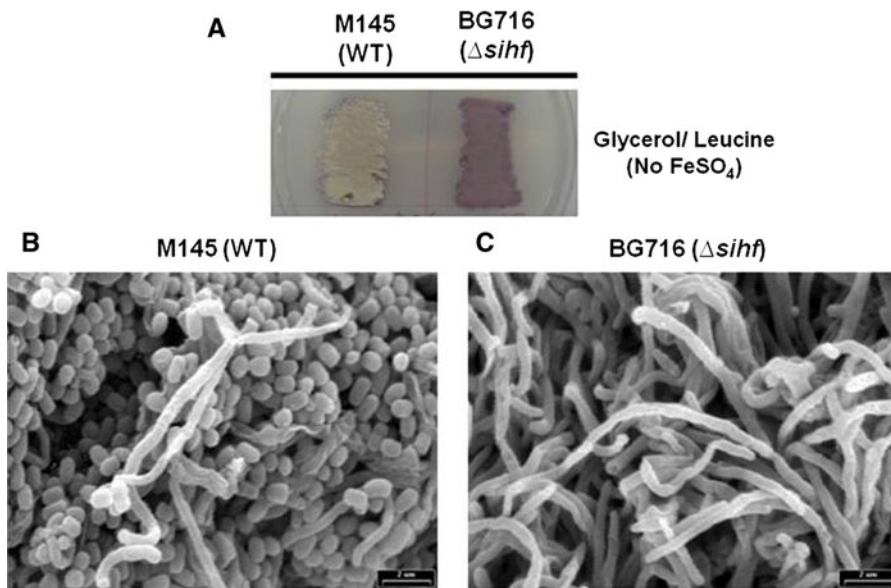
mutant BG716 (for the promoter, 223 bp upstream of SCO1480 was cloned together with SCO1480). Another complementation strain was made by using plasmid pYH81 which expresses *sihf* from the constitutive *ermE*\* promoter. Antibiotic production on glucose/glycine minimal media was then examined. While M145 produced little pigmented antibiotics (ACT or RED) under these conditions, the *sihf* null mutant BG716 produced significant amounts of ACT and RED; however, antibiotic production was reduced again following complementation by constructs expressing *sihf* in both ways (BG7161 and BG7165). These complementation experiments strongly suggest that the enhanced production of ACT was solely due to the deletion of *sihf* (Fig. 3b). Antibiotic production was quantitatively measured and BG716 produced approximately 1.4-fold more ACT and 1.9-fold more RED as compared to the parent M145 (Fig. 3c, d).

### sIHF is required for proper sporulation and for spore integrity

To analyse role of sIHF in the control of development, *S. coelicolor* M145 and its *sihf* null mutant BG716 were grown on minimal media agar plates containing various carbon and nitrogen sources. As a result, BG716 showed strongly delayed and less pronounced

**Fig. 3** Differential antibiotic production of *S. coelicolor* M145 and its *sihf* null mutant BG716 when cultured on various minimal media and R5<sup>−</sup> complex media (a). Complementation of BG716 with SCO1480 (BG7161 and BG7165) grown on minimal media containing glucose and glycine for 6 days. sIHF is cloned in a multi-copy vector with the *ermE*\* promoter (BG7161) and in an integration vector containing the native promoter of *sihf* (BG7165) (b). Measurement of ACT (c) and undecylprodigiosin (d) of M145 and BG716 when cultured on R5<sup>−</sup> media





**Fig. 4** Comparison of sporulation of *S. coelicolor* M145 and its *sihf* null mutant BG716. **a** Both strains grown on  $\text{Fe}^{2+}$ -deficient minimal media containing glycerol and leucine. **b**, **c** Scanning electron micrographs of M145 (**b**) and BG716 (**c**) at  $\times 5,000$

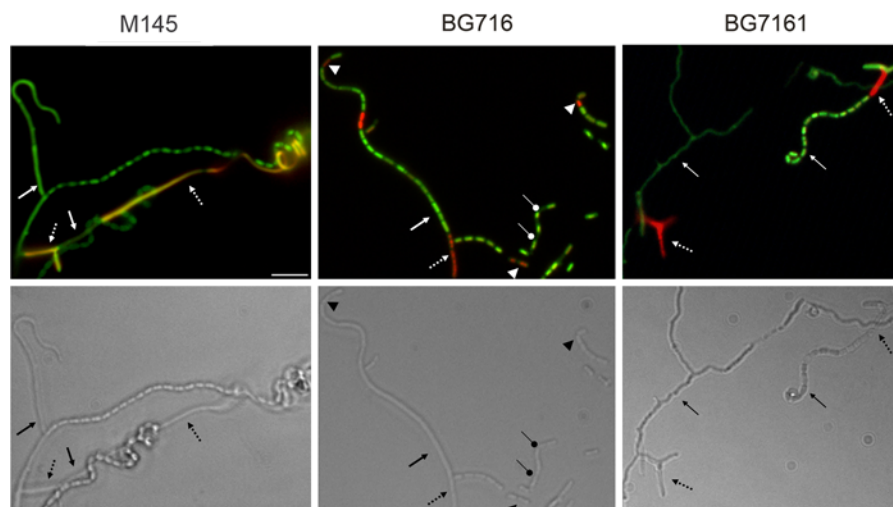
magnification. Note that the *sihf* null mutant fails to sporulate on glycerol/leucine minimal media without  $\text{Fe}^{2+}$  ion, while the parental strain M145 does produce spores under these conditions

sporulation on both MM and R5<sup>−</sup> agar plates. This effect was particularly strong when grown on MM with glycerol and leucine as carbon and nitrogen sources without  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (normally 36  $\mu\text{M}$ ) (Fig. 4a). Surprisingly, while the parental strain M145 showed normal sporulation on these iron-depleted media, BG716 failed to produce spores. Using cryo-scanning electron microscopy (cryo-SEM) the difference in sporulation between M145 and BG716 was clearly visible (Fig. 4b, c), with BG716 hardly producing any spores.

Interestingly, the *sihf* mutant spores had an increased spores size, from  $1.2 \pm 0.2 \mu\text{m}$  for M145 to  $1.5 \pm 0.4 \mu\text{m}$  for the *sihf* null mutant (Fig. 5); the average spore size was restored to the wild-type range of  $1.2 \pm 0.4 \mu\text{m}$  by complementation with wild-type *sihf* (strain BG7161). Additionally, fluorescence microscopy of the chromosome revealed that sIHF did not affect DNA distribution during vegetative growth (Supplementary Fig. 2), but that it is very important for the correct DNA segregation during sporulation. Comparison of the integrity of the aerial hyphae and spores of M145, BG716, and BG7161 by live/dead staining revealed a high proportion of dead cells in the mutant BG716, while hardly any were found in the parental strain M145 (Fig. 5). The viability was

restored when the mutant was complemented with a vector expressing *sihf* (strain BG7161). In *sihf* null mutants a significant proportion of the spores (10.2% of a total of 500 counted spores) were empty, i.e. not containing any DNA, while the DNA content of the spores that did contain DNA measured by the fluorescence intensities varied significantly (Fig. 5; Table 2, *F* test *P* value  $<0.001$ ). Additionally, the percentage of damaged spores (i.e. stained with propidium iodide) increased to 4.3%. In both the parental strain and the complemented mutant BG7161 no empty spores were observed and damaged spores were observed with a frequency of less than 1% (Table 2).

These live/dead imaging data clearly indicate that the hyphae and spores of the mutant are less viable, and we anticipated that they would therefore also be more sensitive to general stresses such as heat shock. Therefore, *S. coelicolor* M145, *sihf* mutant BG716, and a strain with enhanced expression of sIHF (BG7164) were analyzed in a heat inactivation experiment as described in the “Materials and methods” section. After 10 min of incubation at 60°C, most cells of BG716 had died (15% survival based on cfu counts), whereas M145 and BG7164 were much less affected, displaying relatively high viable counts of 50 and 64%, respectively (Fig. 6). After 20 min of heat



**Fig. 5** Live/dead staining (top) of *S. coelicolor* of M145 (left), BG716 (SCO1480 deletion mutant, middle), and BG7161 (complementation strain, right) with their corresponding light images (bottom). Strains were grown on SFM for 4 days. Dashed arrows mark lysing (dead) aerial hyphae, solid arrows mark viable aerial hyphae. Arrowheads mark dead spores and

balloon heads mark empty spores. Note that these are only observed in the SCO1480 deletion strain, whereas viable and dead aerial hyphae occur both in wild type and BG7161 as well as BG716. For statistics of live/dead spores and spore sizes see Table 2. Bar, 5  $\mu$ m

**Table 2** Live/dead staining and size distribution of the spores

|                    | M145          | BG716         | BG7161        |
|--------------------|---------------|---------------|---------------|
| Live/dead staining |               |               |               |
| Live               | 99.8%         | 85.5%         | 99.6%         |
| Dead               | 0.2%          | 4.3%          | 0.4%          |
| Empty              | 0.0%          | 10.2%         | 0.0%          |
| Spore size         |               |               |               |
| Size ( $\mu$ m)    | $1.2 \pm 0.4$ | $1.5 \pm 0.2$ | $1.2 \pm 0.2$ |

For each strain 500 spores were counted. See also Fig. 5

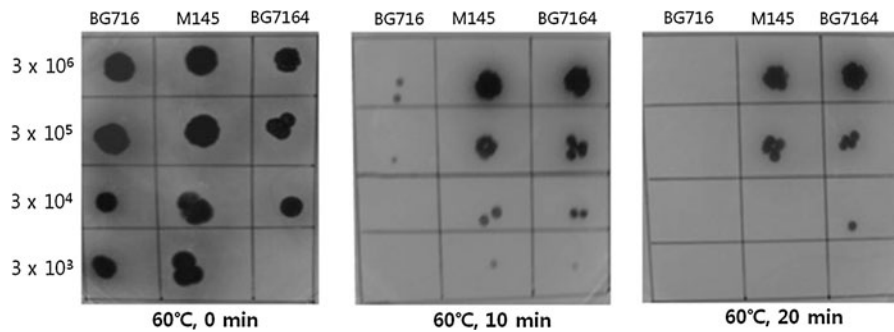
treatment, no survival was observed for BG716, whereas for M145 and BG7161 a survival was found of 26 and 57%, respectively. The higher survival for BG7161 was reproducible. When the viability test was done with the spores, the survival rate of BG716 was reduced more than half compared to that of mycelia (data not shown). These results suggest that although *sihf* is not essential, it is very important for governing cell viability.

#### Proteome analysis of the *sihf* null mutant

To obtain more insight into the physiological role of *sihf*, we compared the protein expression profiles of the mutant and the parental strain using comparative 2D-gel

analysis. For this, M145 and BG716 were cultured on MM agar plates with glucose and glycine (MMGG) for 5 days. MMGG was chosen because BG716 showed distinctive differences in antibiotic production and morphology on this media as compared to M145. Differentially expressed proteins (>1.5 fold difference and at least 3 peptides identified by mass spectrometry) are listed in Tables 3A and B. Glycine was added, as it is one of the precursors for RED biosynthesis in *S. coelicolor*. As expected, BG716 produced more RED compared to M145 when they were grown in MMGG. Glycine-utilizing pathways were up-regulated in BG716, such as serine hydroxymethyltransferase (SCO4837), which converts glycine into serine, and phosphoserine aminotransferase (SCO4366), which is involved in converting serine into 3-phosphoglycerate, a glycolytic intermediate. This suggests that enhanced RED production in BG716 may be caused by the elevated expression levels of glycine-utilizing pathways.

Interestingly, SCO5080 (*ActVA5*) was also enhanced in the mutant, in line with the enhanced ACT production in BG716. In addition, antibiotic repressor CutR (SCO5862) was down-regulated in BG716. The two-component signal transduction system CutRS of *S. lividans* which is known to be closely related to *S. coelicolor*, was reported to repress antibiotic production in both *S. lividans* and



**Fig. 6** Viability test of M145, BG716 (SCO1480 deletion mutant), and BG7164 (M145 with *sihf* behind  $p_{\text{permE}}$  on a multi-copy vector) after the heat treatment at 60°C for 0, 10, and 20 min. Strains were cultured in 25 ml R5<sup>−</sup> liquid media for 20 h, and the cells were harvested and taken the same amount of

cfus. Note the strongly enhanced sensitivity of the submerged mycelia to high temperatures, with viability more than two orders of magnitude lower than of mycelia from the parental strain M145 and of BG7164

*S. coelicolor* (Chang et al. 1996). Therefore, the reduced protein level of CutR again correlates well to the increased antibiotic production in BG716.

Five putative transcriptional regulators (SCO0132, SCO5862, SCO1654, SCO5811 and SCO7785) were found to be differentially expressed in BG716 (Table 3). Furthermore, the YidC protein (SCO3883), which is part of the secretion machinery and assists in the integration, folding, and assembly of inner membrane proteins into the membrane (Luirink et al. 2005), showed three-fold reduction in expression in the *sihf* mutant, while the cytoskeletal protein FilP (Bagchi et al. 2008) could not be identified in the mutant and was readily identified in the parental strain M145. These major changes highlight the global role for *sihf*. In the previous studies on deletion mutants of more specific transcriptional regulators, only one or two transcriptional regulators were found to be differentially expressed in comparative 2D-gel analysis (Yang et al. 2009, 2010). Our results suggest that *siHF* may govern several lower-level transcriptional regulators, and act as a global and upper-level regulator in the hierarchical transcriptional network, along with other important global regulators like DasR (Rigali et al. 2006, 2008) and BldD (den Hengst et al. 2010). All of these regulators have a major effect on both development and antibiotic production.

## Discussion

Global control of cell-cycle and antibiotic regulators is controlled by distinct classes of regulatory elements in

streptomycetes. Their saprophytic life style and complex life cycle necessitates complex transcriptional control, exerted by well-studied regulators such as AdpA (which responds to the  $\gamma$ -butyrolactone A-factor), the cAMP receptor protein CRP (pleiotropic regulator of germination and early growth), the nutrient sensor DasR (switch to development), the global amino acid regulator NdgR and the developmental Bld and Whi regulators (Bibb 1996; Flardh and Buttner 2009; Ohnishi et al. 2005; Piette et al. 2005; Rigali et al. 2006; van Wezel et al. 2009; Yang et al. 2009). A second category of regulators globally affects gene regulation at the level of translation, most notably the rare leucyl-tRNA BldA, which relates to developmental control of TTA-containing genes (Lawlor et al. 1987), and tmRNA, which is essential for the translation of antibiotic- and stress-related genes such as *dnaK*, *dasR*, *ssgRA* and *tipA* (Barends et al. 2010).

Contrary to these DNA-binding regulators, only a few studies were exerted to the understanding of the basic architectural elements in streptomycetes. SCO2950 was revealed to be a conventional nucleoid-associated HU protein that is similar to *E. coli* HU $\alpha$  and HU $\beta$ , whereas the two-domain HU protein SCO5556 exists only in actinobacteria (Salerno et al. 2009). SCO5556 is specifically expressed during sporulation, while SCO2950 is active during vegetative growth. Spores of a SCO5556 deletion mutant had an increased average nucleoid size as compared to the parental strain and were defective in heat resistance (Salerno et al. 2009). In other species such as *E. coli*, these proteins are known to be involved in the control

**Table 3** Lists of differentially expressed proteins in BG716

| Spot ID                                                                                       | Annotation                                                                 | SCO #   | Peptide hits | Different peptides | Fold change |
|-----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|---------|--------------|--------------------|-------------|
| (A) Proteins that were underrepresented (more than 1.5-fold) in <i>sihf</i> null mutant BG716 |                                                                            |         |              |                    |             |
| 1318                                                                                          | FumC, fumarate hydratase C                                                 | SCO5042 | 247          | 9                  | 1.7         |
| 1329                                                                                          | Dxr, 1-deoxy-D-xylulose 5-phosphate reductoisomerase                       | SCO5694 | 116          | 6                  | O           |
|                                                                                               | XylB, xylulose kinase                                                      | SCO1170 | 16           | 3                  |             |
| 1343                                                                                          | SucC, succinyl-CoA synthetase beta chain                                   | SCO4808 | 25           | 4                  | 9           |
| 1395                                                                                          | Gap2, glyceraldehyde 3-phosphate dehydrogenase                             | SCO7511 | 124          | 5                  | O           |
| 1414                                                                                          | Mdh, malate dehydrogenase                                                  | SCO4827 | 19           | 2                  | 3           |
|                                                                                               | Putative inner membrane protein translocase component YidC                 | SCO3883 | 9            | 2                  |             |
| 1415                                                                                          | Cytoskeletal protein FilP                                                  | SCO5396 | 39           | 4                  | O           |
| 1499                                                                                          | Pgm, phosphoglyceromutase                                                  | SCO4209 | 5            | 2                  | 2           |
|                                                                                               | InfC, translation initiation factor IF-3                                   | SCO1600 | 4            | 2                  |             |
| 1531                                                                                          | Probable transcriptional regulator                                         | SCO0132 | 4            | 2                  | 1.5         |
|                                                                                               | Beta-xylosidase                                                            | SCO0293 | 5            | 2                  |             |
| 1539                                                                                          | RNA polymerase sigma factor                                                | SCO2639 | 4            | 2                  | O           |
| 1542                                                                                          | CutR, two-component regulator CutR                                         | SCO5862 | 24           | 6                  | 2.1         |
|                                                                                               | Putative two-component response regulator                                  | SCO1654 | 4            | 2                  |             |
| 1546                                                                                          | Transcriptional regulator                                                  | SCO5811 | 4            | 2                  | 1.5         |
| 1553                                                                                          | Tellurium resistance protein                                               | SCO4277 | 39           | 2                  | 1.5         |
| 1433                                                                                          | HisA, phosphoribosyl isomerase A                                           | SCO2050 | 93           | 6                  | 1.8         |
| 1591                                                                                          | Hypothetical protein                                                       | SCO7269 | 82           | 3                  | O           |
| 1615                                                                                          | RplL, 50S ribosomal protein L7/L12                                         | SCO4653 | 5            | 2                  | O           |
| (B) Proteins that were overrepresented (more than 1.5-fold) in <i>sihf</i> null mutant BG716  |                                                                            |         |              |                    |             |
| 1214                                                                                          | GlyA, serine hydroxymethyltransferase                                      | SCO4837 | 94           | 6                  | O           |
|                                                                                               | PdhL, dihydrolipoamide dehydrogenase                                       | SCO2180 | 7            | 2                  |             |
| 1253                                                                                          | Acetyl-CoA acetyltransferase                                               | SCO3079 | 105          | 7                  | 2.2         |
|                                                                                               | Dipeptidase                                                                | SCO3058 | 5            | 2                  |             |
| 1254                                                                                          | Phosphoserine aminotransferase                                             | SCO4366 | 52           | 3                  | 1.8         |
| 1270                                                                                          | Hydrolase (ACT cluster)                                                    | SCO5080 | 4            | 2                  | 2.3         |
| 1273                                                                                          | Gap1, glyceraldehyde-3-phosphate dehydrogenase                             | SCO1947 | 221          | 8                  | 2.2         |
| 1339                                                                                          | Oxidoreductase                                                             | SCO4266 | 238          | 4                  | O           |
|                                                                                               | Transcriptional regulator                                                  | SCO7785 | 7            | 2                  |             |
| 1346                                                                                          | PcrA, 20S proteasome alpha-subunit                                         | SCO1643 | 29           | 2                  | 1.8         |
| 1380                                                                                          | TpiA, triosephosphate isomerase                                            | SCO1945 | 125          | 5                  | O           |
| 1474                                                                                          | Decarboxylase                                                              | SCO2822 | 28           | 2                  | O           |
|                                                                                               | Nucleotide binding protein                                                 | SCO4614 | 4            | 2                  |             |
| 1475                                                                                          | MoaB, molybdenum cofactor biosynthesis protein (putative secreted protein) | SCO3179 | 7            | 2                  | 2.8         |
| 1483                                                                                          | CvnA9, integral membrane protein                                           | SCO1630 | 4            | 2                  | O           |
| 1496                                                                                          | Secreted protein                                                           | SCO1860 | 52           | 2                  | O           |
| 1498                                                                                          | PanD, L-aspartate-alpha-decarboxylase                                      | SCO0978 | 89           | 2                  | O           |
| 1401                                                                                          | Map2, methionine aminopeptidase                                            | SCO6409 | 79           | 3                  | 1.7         |

Spots that were only present in BG716 or M145 gels were indicated as O

of many operons by either affecting the DNA architecture or transcription (Goosen and van de Putte 1995). Architectural proteins are more likely to globally affect transcription, which affect other regulators by interfering with or assisting in the binding of regulators at the same binding site (Devroede et al. 2006).

mIHF was first found in *M. smegmatis* and *Mycobacterium tuberculosis*, where it stimulates the integration of mycobacteriophage L5 (Pedulla et al. 1996). Many homologs were found in the genomes of mycobacteria, corynebacteria, streptomycetes, and rhodococci. In mycobacteria, mIHF is essential for survival and while involvement in secondary metabolism was not yet reported, the strong increase in concentration of mIHF at the stationary phase suggests its involvement in processes relating to secondary metabolism and stationary events (Pedulla and Hatfull 1998). In the life cycle of streptomycetes this phase corresponds to antibiotic production and morphological differentiation.

More information on the general function of this important gene in *Mycobacterium* may be gleaned from the phenotype of the *sihf* mutant in *S. coelicolor*, although some caution should be taken as *Mycobacterium* and *Streptomyces* are rather distantly related members of the family of actinomycetales. In previous studies, IHFs were shown to reach their highest intracellular levels just prior to the stationary phase, and were known to be involved in the regulation of genes required for the establishment of the stationary phase (Aviv et al. 1994; Ditto et al. 1994; Hengge-Aronis 1996). In addition, mIHF is most abundant prior to entry into the stationary phase even though its effect is weaker than of other IHFs. It is also proposed to regulate the expression of stationary phase-specific genes similar to the *E. coli* IHF (Pedulla and Hatfull 1998). The effect on sporulation and antibiotic production indeed seems to support a dominant role for sIHF in the control of stationary phase-specific gene regulation in streptomycetes.

In support of this growth phase-dependence, we observed major changes in spore morphology and integrity. The average spore size increased by around 25%, from an average of 1.2  $\mu\text{m}$  for the parental strain and the complemented mutant to 1.5  $\mu\text{m}$  for the *sihf* null mutant. Live/dead staining further demonstrated significant changes in spore integrity and DNA distribution. While no significant changes were observed

during vegetative growth, around 10% of the *sihf* mutant spores were empty, i.e. not containing any DNA, while some 4% damaged or dead spores (i.e. stained with propidium iodide) were found (Table 2). This demonstrates that sIHF becomes more important for integrity of the cells and proper DNA segregation at later stages of the *Streptomyces* life cycle.

The onset of antibiotic production and sporulation are highly coordinated (Bibb 2005; van Wezel and McDowall 2011), with antibiotic production typically coinciding with growth cessation in response to the accumulation of ppGpp (Chakraborty and Bibb 1997; Strauch et al. 1991). The occurrence of many mutants deficient in both antibiotic production and the formation of aerial hyphae suggests that these processes are partially controlled by the same set of regulators (Bibb 2005; Chater 2001; Rigali et al. 2008; Uguru et al. 2005). Our data show that sIHF functions in a distinctly different manner, repressing antibiotic production and ensuring correct and abundant sporulation. Proteomic data show down-regulation of the antibiotic repressor CutR, membrane assembly protein YidC, and cytoskeletal protein FilP in BG716, which suggests that sIHF acts as a repressor of antibiotic production and plays a role in the control of morphological differentiation. Although sIHF could be affected by different nutrients, this is a rare example of a regulatory gene oppositely governing the two main developmental events. Thus it appears that sIHF has more complex and versatile effects than originally expected in terms of the control of chemical and morphological differentiation.

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