Evidence for the Formation of ScbR/ScbR2 Heterodimers and Identification of One of the Regulatory Targets in *Streptomyces coelicolor*

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Abstract

The homologous transcriptional regulators ScbR and ScbR2 have previously been identified as γ-butyrolactone (GBL) and antibiotic receptors, respectively. They regulate diverse physiological processes in *Streptomyces coelicolor* in response to GBL and antibiotic signals. In this study, ScbR and ScbR2 proteins were shown to interact using a bacterial two-hybrid system where adenylate cyclase activity was reconstituted in *Escherichia coli* BH101. These ScbR/ScbR2 interactions in *S. coelicolor* were then demonstrated by co-immunoprecipitation. The ScbR/ScbR2 heterodimer was shown to co-exist with their ScbR and ScbR2 respective homodimers. When potential operator targets in *S. coelicolor* were investigated, the heterodimer was found to bind in the promoter region of *sco5158*, which however was not a target for ScbR or ScbR2 homodimers. These results revelaed a new mechanism of regulation by ScbR and ScbR2 in *S. coelicolor*.

Key Words: ScbR, ScbR2, protein interaction, heterodimer, *sco5158*

Introduction

Streptomycetes are soil-dwelling Gram-positive bacteria with exceptional secondary metabolite producing capability and complex morphological development cycle. All these events are controlled by precise and sophisticated regulatory systems, among which transcriptional regulators are the main players. In sequenced *Streptomyces* genomes, regulators encoding genes account for approximately 12% of the chromosomal genes (Bentley et al. 2002). They typically control gene expression in the form of one or two-component systems in response to environmental cues (Romero-Rodriguez et al. 2015). Crosstalks between the activity of regulators encoded within different secondary metabolite gene clusters have been established and result in complex regulatory networks in *Streptomyces* (Li et al. 2015; Liu et al. 2013b).

The *Streptomyces coelicolor* protein ScbR is known to be a γ-butyrolactone (GBL) receptor that represses the transcription of its own gene and that of *scbA*, which

encodes the GBL synthase (Takano et al. 2001). In addition ScbR regulates other aspects of *S. coelicolor* primary and secondary metabolism by interacting with additional DNA targets (Li et al. 2015; Takano et al. 2005). ScbR2 serves as the antibiotic receptor and modulates *S. coelicolor* behaviours in response to antibiotic signals (Wang et al. 2014; Xu et al. 2010). ScbR2 was also recently shown to control additional cellular events, especially secondary metabolism in *S. coelicolo* (Li et al. 2015). ScbR and ScbR2 are highly homologous (50% similarity over 194 amino-acids) and have been shown to interact with common DNA binding sites (Li et al. 2015). ScbR2 is the key regulator turning off the synthesis of GBL and coelimycin antibiotics by binding to the promoter regions of *scbA* and *kasO,* which are two known targets of ScbR (Gottelt et al. 2010; Wang et al. 2011). In addition to regulating these specific pathways, ScbR and ScbR2 both function as pleiotropic regulators in *S. coelicolor* and regulate multiple cellular events. Interestingly ScbR is mainly produced during the early growth phase while ScbR2 starts its expression at a later time (Li et al. 2015).

Both ScbR and ScbR2 belong to the TetR regulators family, proteins of which comprise a conserved helix-turn-helix binding motif in the N-terminus sequence and a highly variable ligand binding pocket in the C-terminus. This latter feature confers TetR-family regulators the ability to respond to a vast diversity of environmental stimuli (Ahn et al. 2012). Structural studies revealed that TetR-family regulators interact with their DNA binding sequences in the form of homodimers (Hillen and Berens 1994; Hinrichs et al. 1994). With the exception of artificially designed TetR-related heterodimers (Stiebritz et al. 2010), to date no natural heterodimer has been observed among TetR family regulators. However, the formation of heterodimers involving homologous proteins is a well-known phenomenon in both the eukaryotic and prokaryotic worlds. Importantly the regulatory role of such heterodimers cannot usually be accomplished by the corresponding homodimers (Morimoto et al. 2015; Parmentier 2015). In *S. coelicolor*, the formation of the EsxA/EsxX heterodimer (proteins of the WXG-100 superfamily) was reported to be involved in the regulation

of sporulation (Akpe San Roman et al. 2010) while the WhiI/BldM heterodimer (proteins of the atypical response regulator family) were shown to regulate development in Streptomycetes (Al-Bassam et al. 2014). MmfR and MmyR, that are ScbR and ScbR2 homologues respectively, are both encoded in the *S. coelicolor* linear plasmid SCP1 and were speculated to form a heterodimer involved in controlling the production of methylenomycin furans (analogous to GBLs) and methylenomycin antibiotics (O'Rourke et al. 2009) but no evidence has been shown. The possibility of ScbR and ScbR2 to form heterodimer was therefore investigated in this study, since Based on our previous work that revealed some common DNA binding sites as well as exclusive targets for ScbR and ScbR2 homodimers, in this study we aimed to investigate the formation and the role of a putative ScbR/ScbR2 heterodimer (Li et al. 2015).

Materials and Methods

Strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1 and the oligonucleotides used as primers are listed in Supplemental Table S1. *S. coelicolor* strains were incubated on MS solid medium for sporulation and in liquid SMM medium for mycelium harvest. *E. coli* strains were grown in Luria–Bertani (LB) medium containing ampicillin (100 μg/mL), kanamycin (50 μg/mL), apramycin (50 μg/mL), hygromycin (50 μg/mL), streptomycin (50 μg/mL) or chloramphenicol (50 μg/mL) when necessary.

Plasmids construction

To generate plasmids pKT25-ScbR, pUT18-ScbR2, pUT18-ScbR and pUT18-ScbR2t, *scbR*, *scbR2* and truncated *scbR2* gene fragments were amplified from genomic DNA of *S. coelicolor* with primers scbR-25F/scbR-25R, scbR2-18F/scbR2-18R, and scbR2-18F/scbR2t-18R, respectively, digested with *Xba*I and *Xho*I restriction enzymes, and then ligated into the pKT25linker and pUT18Clinker. To construct plasmid pIJ10500::scbR2, fragment harboring *scbR2* gene along with its native

promoter was amplified from the genome with primer R2flagF/R2flagR, digested with *Spe*I and *Xho*I, and ligated with pIJ10500 processed with the same enzymes. Plasmid pIJ10500::scbR2 was introduced into *ΔscbR2* to acquire strain scbR2-Flag. For co-expression of ScbR and ScbR2, primers RcoF/RcoR, and R2coF/R2coR were used to amplify *scbR* and *scbR2* genes from the genome and digested with *Eco*RI/*Hin*dIII and *Nde*I/*Avr*II,respectively, and then they were inserted into pCDFDuet-1 to generate plasmids pCDFDuet-ScbR, pCDFDuet-ScbR2 and co-expression plasmid pCDFDuet-ScbR-ScbR2.

Bacterial two-hybrid system

The bacterial two hybrid system (BATCH) is based on the reconstitution of the activity of adenylate cyclase, which is responsible for the synthesis of cyclic adenosine 3,5′ -monophosphate (cAMP) and thus activating the transcription of the *lac* operon in cells. *E. coli* BTH101, a mutant strain where the coding gene for the endogenous adenylate cyclase has been deleted (*cya*$^{-}$), was used as the host for BATCH. When the proteins of interest were fused to the T25 or the T18 domains of adenylate cyclase, the adenylate cyclase activity was expected to be restored only if these proteins interacted in the *cya*$^{-}$ strain, bringing T18 and T25 domains together. Two plasmids carrying the T25 and T18 fusions were transformed into 100 µl of *E. coli* BTH101 competent cells and incubated on ice for 30 min. The cells were then heat shocked at 42 °C for 90 s, followed immediately by 2 min on ice. Then, 1 ml LB was added and the cells were incubated at 37 °C with shaking for recovery for 1 h. 500 µl of the culture were then plated on LB plates containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Plates were incubated at 30 °C for 48 h. Single colonies were then picked from the plates and grown in liquid LB containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.5 mM isopropyl-β-dithiogalactopyranoside (IPTG) overnight at 30 °C with shaking. The next day, 2 µl of each culture was dropped on LB plates supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml), X-Gal (40 µg/ml), and IPTG (0.5 mM).The plates were then incubated at 30 °C until a blue coloration appeared (Battesti and Bouveret 2012).

Co-immunoprecipitation (CO-IP)

Strain scbR2-Flag was cultured in SMM liquid medium for 30 hours and mycelium was harvested by centrifugation at 12000 rpm for 10 min at 4 °C. 1 g of the pellet was resuspended in 8 ml lysis buffer [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10% glycerol, 0.1% Triton X100, protease inhibitor (complete mini, Roche, Basel, Switzerland)] and sonicated at 5 s/5 s at 200 W to clearness. After centrifugation at 12000 rpm for 10 min at 4 °C, anti-FLAG antibody (Sigma, MO, USA) was added to the cell extract at a concentration of 0.5 μg per 1 mg total protein and incubated with protein A beads at 4 °C for 4 hours. Then beads enriched with coimmunoprecipitate were then collected and washed using lysis buffer (without glycerol) for three times, resuspended in 100 μl 2xSDS loading buffer. After centrifugation, the supernatant was subjected to western blotting analysis.

Western blotting

Western blotting was used to detect the presence of ScbR in CO-IP immunoprecipitates. Samples were denatured at 95°C for 10 minutes, and 5 μg total protein samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene fluoride membranes (Millipore Inc, Darmstadt, Germany), and nonspecific binding was blocked by using a 5% bovine serum albumin (BSA) solution for 1 hour at room temperature. The membranes were washed three times and probed with primary anti-ScbR antibody (CoWin Biotech Co. Ltd, Beijing, China). After washing three times, the proteins were visualized using horseradish peroxidase conjugated secondary antibodies (Santa Cruz, CA, USA) and an enhanced chemiluminescence system (CoWin Biotech Co. Ltd, Beijing, China).

Co-expression of ScbR and ScbR2

Plasmids pCDFDuet-ScbR, pCDFDuet-ScbR2 and pCDFDuet-ScbR-ScbR2 were expressed in *E. coli* C41(DE3) at 4 °C overnight after induction with 0.5 mM IPTG. His_6-ScbR was purified with Ni column, while ScbR2 with a Strep tagII was purified

with Strep-Tactin superflow column (IBA, Göttingen, Germany) following manufacturer instruction. For the purification of the ScbR-ScbR2 complex, cell extract was first loaded into Ni column pre-equilibrated with PBS (pH 7.4) buffer with 10 mM imidazole for His-ScbR enrichment, washed with 50 volume of buffer [PBS (pH 7.4), 60 mM imidazole], and then eluted with buffer [PBS (pH 7.4), 300 mM imidazole]. The eluted protein was re-loaded to Strep-Tactin column pre-equilibrated with PBS (pH 7.4), washed with 20 volume of PBS (pH 7.4), and eluted with buffer [PBS (pH 7.4), 2.5 mM desthiobiotin]. After consecutive Ni column and Strep-Tactin column affinity purification, only ScbR-ScbR2 complex with both tags could be enriched.

Size Exclusion Chromatography

ScbR, ScbR2 and the ScbR/ScbR2 complex were concentrated down to 1~2 mg/ml for size exclusion chromatography (SEC) analysis. SEC was carried out using a TSKgel G3000SWxl column (Tosoh Bioscience LLC, PA, USA) and eluted at 0.5 ml/min in mobile buffer (100 mM PBS, pH 8.0, 0.1 M Na_2SO_4). The proteins injected in the system were detected using a UV detector set to detect absorbance at 280 nm. Three protein standards, bovine serum albumin (67, 134 kDa), AlpJ (27 kDa) and lysozyme (14.4 kDa) were used to estimate the molecular mass of ScbR, ScbR2 and that of the ScbR/ScbR2 complex.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described in our previous study (Wang et al. 2011). The DNA probe (6 ng) was mixed with varying amount of protein in a buffer containing 20 mM Tris-base (pH 7.5), 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.5 μg/μl calf BSA and 5% (v/v) glycerol in a total volume of 20 μl, and incubated at 25 °C for 20 min. After incubation and electrophoresis, the non-denaturing 4% (w/v) polyacrylamide gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, MA, USA) for 30 min in TBE (89 mM Tris-base, 89 mM boric acid, 1 mM EDTA, pH 8.0) buffer, and photographed

under blue transillumination using Darker Reader (Clare Chemical, CO, USA).

Results

Molecular interactions between ScbR and ScbR2 proteins

To assess if ScbR and ScbR2 proteins interacted, ScbR and ScbR2 were fused to the T25 and T18 domains of an adenylate cyclase, respectively. The resulting plasmids pKT25-ScbR and pUT18-ScbR2 were then co-transformed into *E. coli* BTH101 for BACTH analysis (Battesti and Bouveret 2012). On the basis that TetR family transcriptional regulators normally form homodimers, BTH101 was also co-transformed with plasmids pKT25-ScbR and pUT18-ScbR and this strain used as a positive control. As the crystal structure of TetR had revealed that the α8 and α10 helices at the C-terminus are critical for dimerisation (Kisker et al. 1995), a truncated version of ScbR2 (ScbR2t), in which the amino-acid sequence from helix α8 to helix α10 was removed, was also fused to the T18 domain of adenylate cyclase. Plasmids pUT18-ScbR2t and pKT25-ScbR were then co-transformed into BTH101 and the resulting strain used as a negative control. As shown in Fig. 1a, the strain co-expressing ScbR and ScbR2 appeared blue on the LB plate containing X-Gal, indicating that adenylate cyclase activity had been reconstituted. A similar activity was observed on the positive control strain but was absent when ScbR2t was used in place of ScbR2. The results of these BATCH analyses in *E. coli* therefore revealed that ScbR and ScbR2 do interact *in vivo*.

To further validate the interaction between ScbR and ScbR2 *in vivo*, CO-IP assays were conducted using *S. coelicolor*. A pIJ10500::scbR2 plasmid was constructed, in which *scbR2* was expressed under the control of its native promoter and a Flag tag was fused at the the C-terminus of the ScbR2 protein. When introduced into the *S. coelicolor ΔscbR2* strain, this plasmid was able to restore the phenotypic defects caused by the *scbR2* deletion (Supplemental Fig. S1). An anti-Flag antibody was then used to prepare protein samples from M145 and scbR2-Flag strains by CO-IP. The anti-Flag antibody was expected to bind ScbR2 fused to the Flag tag and CO-IP

would permit isolation of any protein interacting with ScbR2 in cell-free extracts prepared with *S. coelicolor* mycelia. The immunoprecipitated proteins were then subjected to SDS-PAGE analysis and the presence of ScbR was detected by western blotting using an anti-ScbR antibody. In parallel, a recombinant ScbR-His_6 protein had been produced and purified from *E. coli* C41(DE3). This protein was included as a positive control in the western blot analyses. Importantly our previous work had established that the anti-ScbR antibody is highly specific for ScbR and does not bind to ScbR2 (Li et al. 2015). We can therefore conclude that the positive signal observed in the CO-IP sample from scbR2-Flag but absent from that of M145 (Fig. 1b) corroborate the presence of a ScbR-ScbR2 interacting complex *in vivo*.

Formation of a heterodimeric ScbR/ScbR2 complex

To determine the *in vivo* interactions between ScbR and ScbR2 in more details, ScbR and ScbR2 were co-expressed in *E. coli* C41(DE3) using the plasmid pCDFDuet-ScbR-ScbR2. The His_6-ScbR/ScbR2-Strep TagII complex could then be isolated by consecutive Ni and Strep-Tactin affinity columns and its molecular mass determined by SEC. The predicted molecular masses of His_6-ScbR and ScbR2-Strep TagII were 25.1 and 24.7 kDa, respectively and SDS-PAGE analyses (Fig. 2a) revealed that equal amounts of recombinant ScbR and ScbR2 were detected in the *E. coli* cell-free extracts. ScbR was then greatly enriched following the Ni column purification but a small fraction of ScbR2 was present in the eluted sample. Finally both bands, corresponding to His_6-ScbR and ScbR2-Strep TagII, were observed after consecutive Ni and Strep-Tactin affinity purifications, confirming the presence of both ScbR (upper band) and ScbR2 (lower band) in the heterodimeric complex. However, the amount of ScbR2 protein detected after the consecutive purifications of the complex was greater than that of ScbR, implying that ScbR and ScbR2 may not simply form a 1:1 heterodimeric complex. Analyses of the purified complex on SEC were revealed two main peaks. The peak eluted last (between 19-20 min.) was also present in samples containing ScbR2 alone or ScbR alone (Fig. 2b). This result therefore demonstrated the presence ScbR2 and/or ScbR homodimers in the sample

containing the ScbR-ScbR2 complex. According to the standard curve of molecular mass, the time at which the ScbR and ScbR2 homodimers eluted should correspond to a 42 kDa protein/protein complex, which was not consistent with the predicted ~50 kDa for ScbR or ScbR2 homodimers. However, this discrepancy could be explained by the fact that protein shape and charge also effect retention time and therefore the apparent molecular weight on SEC analyses (Liu et al. 2013a). Consequently, the calculated molecular weight on SEC is often not an integral multiple of the expected number of monomers. The 51.6 kDa peak, eluted first, was consistent with the theoretical molecular mass of the ScbR/ScbR2 heterodimer (Fig. 2b); suggesting that the heterodimer was also present in the sample. Taken together our results implied that the interactions between ScbR and ScbR2 proteins yield to the formation of an heterodimeric complex that appear to co-exist with the corresponding homodimers. These results also suggest that conversion between ScbR/ScbR2 heterodimers, ScbR homodimers and ScbR2 homodimers may be very dynamic, explaining the skewed stoichiometry of ScbR and ScbR2 after consecutive elutions, as shown in Fig. 2a.

As ScbR and ScbR2 share a high degree of amino-acid sequence similarities, the residues involved in homodimer and heterodimer formations might be conserved. Among all GBL receptor homologues, CprB is the only known protein for which the 3D structure has been solved (Natsume et al. 2003). The similarity of CprB with ScbR is ca. 27% with its similarity with ScbR2 is ca. 29%. Based on the structure of CprB, the dimerisation surfaces in ScbR and ScbR2 were identified using SWISS-MODEL homology modelling (Natsume et al. 2003). The distribution of amino-acids at the dimerisation interface was then analysed using PPsite (Gao et al. 2004). Hydrogen bonds and hydrophobic interactions predicted on the surface of dimeric interaction were significantly different between ScbR homodimers and ScbR2 homodimers (Supplemental Fig. S2). These results suggested that the mechanism and residues involved in ScbR/ScbR2 heterodimeric formation are probably different from those involved in homodimeric formation. This might also provide an explanation about the discrepancies observed between the apparent molecular masses of ScbR or ScbR2

homodimer and that of the ScbR/ScbR2 heterodimer on SEC.

ScbR-ScbR2 heterodimer interacted with the promoter of *sco5158*

Previously, ScbR and ScbR2 were shown to function as homodimers that bind numerous DNA targets (Li et al. 2015). To understand the role of the ScbR-ScbR2 heterodimer, putative binding sequences were searched from the targets of ScbR and ScbR2 homodimers identified by chromosome immunoprecipitation (Li et al. 2015). Amongst these targets captured by ScbR or ScbR2, some did not interact with ScbR or ScbR2 homodimers, and were therefore inferred as potential targets of the ScbR-ScbR2 heterodimer. 17 target promoter regions were amplified from the genome, and subjected to EMSA assays (Xu et al. 2010). Eventually, only the promoter of *sco5158* was found to bind with ScbR-ScbR2 heterodimer (Fig. 3a) and this DNA was not the target of functional ScbR or ScbR2 homodimers; known target sequences of these homodimers were used positive controls (Supplemental Fig. S3). When the promoter sequence of *sco5158* was aligned with that of the *scbA* promoter and that of *kasO* promoter, two known targets of ScbR and ScbR2 (Wang et al. 2011), a possible binding region for the ScbR-ScbR2 heterodimer was extracted (Fig. 3b). Since ScbR and ScbR2 share similar binding motifs (Li et al. 2015), at this stage it is difficult to predict the detailed interactions between the ScbR-ScbR2 heterodimer and the *sco5158* promoter. SCO5158 remains an uncharacterized protein but the genetic organisation of *sco5158* and adjacent genes is quite conserved in *Streptomyces* (Fig. 3c). Based on gene anotations, this set of conserved genes appears to be involved in metal transport in Streptomycetes. The ScbR-ScbR2 heterodimer might regulate metal utilisation in *S. coelicolor* by controlling the expression of *sco5158*.

Discussion

The homologous proteins ScbR and ScbR2 form homodimers that, in response to environmental cues, control gene expression and behaviours in *S. coelicolor*. Despite ScbR and ScbR2 having distinct biological roles, reflected by the different phenotypes of *S. coelicolor ΔscbR* and *ΔscbR2* mutants strains, they share some DNA targets in *S.*

297 *coelicolor* genome. While the ScbR and ScbR2 homodimers were shown to
298 independently interact with these targets, the possibility that they may work together
299 and bind, as a heterodimeric complex, certain DNA motifs was investigated in this
300 study. The formation of a ScbR-ScbR2 heterodimer was established and its binding to
301 the promoter region of *sco5158* was demonstrated. This study reports the first
302 evidence of protein-protein interactions between native homologous TetR receptors.

303 Proteins of the TetR transcriptional regulator family typically act as homodimers and
304 recognise palindromic DNA sequences in the genome. Here, our results demonstrated
305 the existence of a ScbR-ScbR2 heterodimer in *S. coelicolor* but they also revealed the
306 dominance of the ScbR and ScbR2 homodimers over the ScbR-ScbR2 heterodimer in
307 the cell (Fig. 2a). Co-expression of ScbR and ScbR2 followed by consecutive
308 purifications steps of the heterodimeric complex consistently led to a mixture of the
309 heterodimer and the corresponding homodimers. These complexes appeared to
310 interconvert dynamically, which made it difficult to obtain pure ScbR-ScbR2
311 heterodimer (Fig. 2). As ScbR and ScbR2 homodimers interact with similar DNA
312 motifs, the target sequence of the heterodimer in the promoter region of *sco5158* does
313 not resemble typical hybrid DNA motifs recognised by two different transcriptional
314 regulators. Consequently the identification of additional targets for the ScbR-ScbR2
315 heterodimer, using bioinformatic tools, could not be achieved.

316 The C-terminus sequence of TetR family regulators is essential for homodimerisation,
317 especially the α8 and α10 helixes (Kisker et al. 1995). Here, our result implied the
318 C-terminus of ScbR2 is also important for heterodimer formation since truncated
319 ScbR2 could no longer interact with ScbR in BATCH assays (Fig. 1a). Nevertheless,
320 the mode of dimerisation for the homodimers were inferred to be different to that of
321 the heterodimer: amino acids contributing to hydrophobic interactions and hydrogen
322 bonds were found to be distributed differently on the dimerisation surfaces
323 (Supplemental Fig. S2). The results of the modelling work were also supported by the
324 fact that the apparent molecular masses of the homodimers and that of the
325 heterodimer differed significantly on SEC analyses (Fig. 2b). Indeed different

dimerisation interfaces between the homodimers and the ScbR-ScbR2 heterodimer could result in changes in binding affinity (Clark et al. 2006; Kortemme et al. 2004), protein shape and charge, all effecting the apparent molecular masses observed on SEC. Most importantly, higher binding affinities within homodimers compared to the binding affinity of ScbR for ScbR2 is expected to favour the formation of homodimers versus that of the heterodimer in the cells (Fig. 2a). Based on the significance of α8 and α10 helixes in dimerisation, computational protein design could assist the development of ScbR-ScbR2 heterodimers with higher binding affinities between the ScbR and ScbR2 monomers. Such protein complexes could prove to be precious tools to study in the true biological roles of heterodimers.

BldM and WhiI are regulators required for aerial mycelium formation and efficient sporulation septation in *S. coelicolor* (Molle and Buttner 2000; Tian et al. 2007). They are both atypical response regulators and, unlike the canonical response regulators, they lack the conserved residues important for phosphorylation (Hutchings et al. 2004). WhiI and BldM were previously shown to form a heterodimer that controls *Streptomyces* differentiation, exemplifying the possibility of heterodimerisation as a new mode of expanding regulatory capabilities in bacterial cells (Al-Bassam et al. 2014). WhiI served as the auxiliary protein of BldM and conferred BldM the ability to recognise a hybrid binding motif, demonstrating the gain of new function by formation of the heterodimer. While the processes of ScbR and ScbR2 respectively sensing GBL and antibiotic signals trigger physiological changes in *S. coelicolor,* the ScbR-ScbR2 heterodimer could integrate different cell signals and control expression of specific genes. Interestingly the GBL growth signals sensed by ScbR are linked to an early growth phase, while antibiotics, sensed by ScbR2, are typically produced later on and indicate the secondary metabolite production phase of *Streptomyces*. The target of the ScbR-ScbR2 heterodimer reported in this study is predicted to regulate metal transport in *Streptomyces*. There are increasing evidences that metal ions play a significant role in cell signalling, for instance Fe starvation in cells could affect bacterial pathogenicity (Kurushima et al. 2012) or Ca^{2+} signalling in mammalian cells

(Clapham. 2007). The integration of two distinct signals (GBL and antibiotics) by the ScbR-ScbR2 heterodimer may therefore contribute to homeostasis during the transition from exponential phase to stationary phase. A more comprehensive study of the regulatory role of the ScbR-ScbR2 heterodimer and that of other TetR heterodimers would greatly develop our understanding of *S. coelicolor* behaviours in response to environmental stimuli.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant 31570031) and by the United Kingdom Biotechnology and Biological Sciences Research Council Grant BB/M022765/1. We thank Professor Mervyn Bibb (John Innes Centre) for providing plasmid pIJ10500 and Professor Emmanuelle Bouveret (Aix-Marseille University) for providing plasmids pKT25linker and pUT18linker; and thank Dr. Keqiang Fan and Jinmin Gao for help in Figure processing.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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476

477 Figure Legends

478 **Fig. 1 Protein interaction between ScbR and ScbR2** (a) The bacterial two-hybrid
479 system assay of ScbR and ScbR2 on indicator LB plate containing X-gal. Strain
480 harboring homodimer of ScbR was used as a positive control, while that containing
481 ScbR and ScbR2t was used as a negative control. (b) Co-IP of ScbR with ScbR2 in *S.*
482 *coelicolor*. 5 μg total protein from coimmunoprecipitate by anti-Flag antibody from
483 scbR2-Flag and M145 cell extract were analysed by western blot, and the presence of
484 ScbR was detected by anti-ScbR antibody. Recombinant ScbR-His_6 purified from *E.*
485 *coli* C41(DE3) was used for reference.

486 **Fig. 2 Purification and analysis of ScbR-ScbR2 complex** (a) SDS-PAGE analysis
487 of ScbR and ScbR2 after co-expression, Ni column enrichment and consecutive Ni
488 column and Strep-tag® purification. (b) SEC analysis of ScbR-ScbR2 complex,
489 ScbR2 and ScbR. Standard curve of molecular mass was displayed in the upper left.

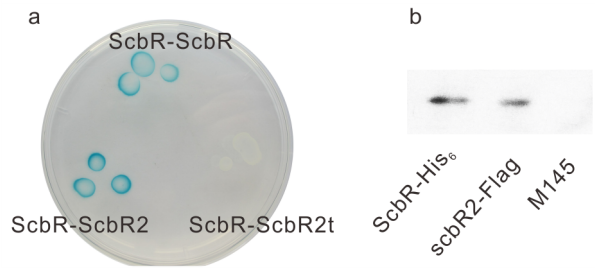
490 **Fig. 3 Target of ScbR-ScbR2 heterodimer**. (a) EMSA assay of *sco5158* promoter
491 with ScbR-ScbR2 complex. 6 ng probe of *sco5158* promoter was incubated with
492 ScbR-ScbR2 complex, ScbR and ScbR2 for interaction. Lanes1-4 contain 0, 60, 120
493 and 240 nM protein in each assay. (b) Predicted binding region of ScbR-ScbR2
494 heterodimer in the promoter of *sco5158*. Sequences were submitted to MEME (Bailey
495 et al. 2009) for motif extraction. (c) Gene organization of *sco5158* and its adjacent
496 genes in the genomes of several *Streptomyces*.

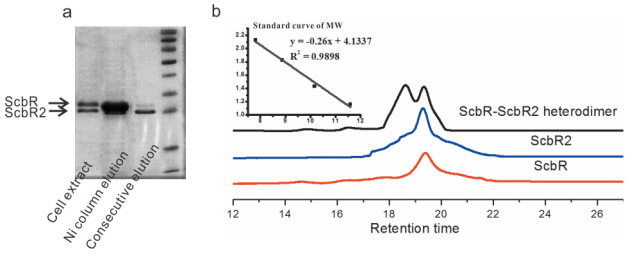
497 **Table 1 Strains and plasmids used in this study**

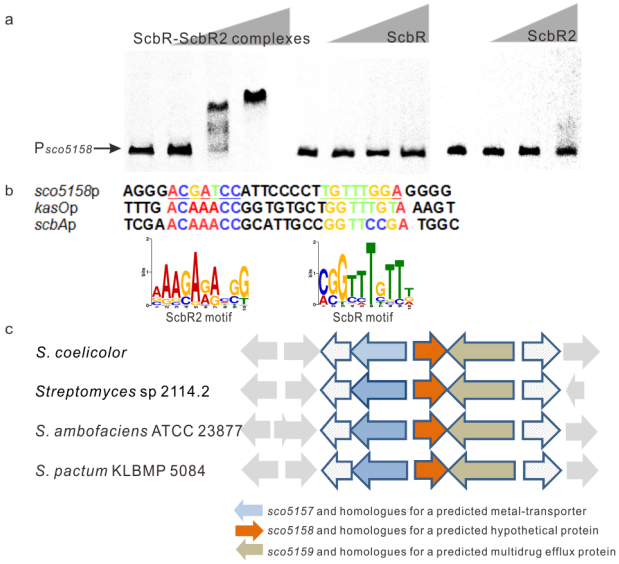
498

Table 1 Strains and plasmids used in this study

Bacterial strains	Relevant genotypes	Source/Reference
S. coelicolor		
M145	Plasmid-free derivative of *S. coelicolor* A3(2)	(Kieser T. 2000)
ΔscbR2	Disruption of *scbR2* gene in M145 background	(Xu et al. 2010)
scbR2-Flag	Genetically complemented ΔscbR2 strain using integrative pIJ10500::scbR2	This study
E. coli		
JM109	General cloning host for plasmid manipulation	Novagen
C41(DE3)	Host for expression plasmids with T7 derived promoter	Novagen
ET12567(pUZ8002)	Donor strain for conjugation between *E. coli* and *Streptomyces*	(Kieser T. 2000)
BTH101	Host for bacterial two-hybrid system, *cya*$^-$	(Karimova et al. 1998)
Plasmids		
pUT18Clinker (pEB355)	Carrying the sequence for the T18 domain of adenylate cyclase, amp^R, ColE1 origin	(Battesti and Bouveret 2012)
pUT18-ScbR	Fused ScbR expression with T18 domain of adenylate cyclase	This study
pUT18-ScbR2	Fused ScbR2 expression with T18 domain of adenylate cyclase	This study
pUT18-ScbR2t	Fused truncated ScbR2 expression with T18 domain of adenylate cyclase	This study
pKT25linker (pEB354)	Carrying the sequence for the T25 domain of adenylate cyclase, kan^R, p15A origin	(Battesti and Bouveret 2012)
pKT25-ScbR	Fused ScbR expression with T25 domain of adenylate cyclase	This study
pIJ10500	Integrated vector, hyg^R, with 3 X Flag tag	(Pullan et al. 2011)
pIJ10500::scbR2	Complementation plasmid with *scbR2* gene in front of 3X Flag-tag	This study
pCDFDuet-1	Co-expression plasmid, Str^R	Novagen
pCDFDuet-ScbR	His_6-ScbR expression plasmid	This study
pCDFDuet-ScbR2	ScbR2-Strep tagII expression plasmid	This study
pCDFDuet-ScbR-ScbR2	Co-expression of His_6-ScbR and ScbR2-Strep tagII	This study


a
ScbR-ScbR
ScbR-ScbR2
ScbR-ScbR2t
b
ScbR-His6
scbR2-Flag
M145


a
ScbR
ScbR2
Cell extract
Ni column elution
Consecutive elution
b
Standard curve of MW
y = -0.26x + 4.1337
R² = 0.9898
ScbR-ScbR2 heterodimer
ScbR2
ScbR
12
14
16
18
20
22
24
26
Retention time


a
ScbR-ScbR2 complexes
ScbR
ScbR2
P sco5158
b
sco5158p AGGGACGATCCATTCCCCTTGTTTGGAGGGG
kasOp TTTG ACAAACCGGTGTGCTGGTTTGTA AAGT
scbAp TCGAACAAACCGCATTGCCGGTTCCGA TGGC
ScbR2 motif
ScbR motif
c
S. coelicolor
Streptomyces sp 2114.2
S. ambofaciens ATCC 23877
S. pactum KLBMP 5084
sco5157 and homologues for a predicted metal-transporter
sco5158 and homologues for a predicted hypothetical protein
sco5159 and homologues for a predicted multidrug efflux protein