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An integrative approach for high-throughput screening and characterization of transcriptional regulators in *Streptomyces coelicolor**

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Abstract: In an age of burgeoning information on genomes and proteomes, determining the specific functions of a gene of interest is still a challenging task, especially genes whose functions cannot be predicted from their sequence information alone. To solve this problem, we have developed an integrative approach for discovering novel transcriptional regulators (TRs) playing critical roles in antibiotic production and decoding their regulatory networks in *Streptomyces* species which contain many regulatory genes for synthesis of secondary metabolites and cell differentiation to spores. The DNA affinity capture assay (DACA) coupled with clustering of DNA chip data was used to find new TRs controlling antibiotic biosynthetic gene clusters. Functions of these newly identified TRs were characterized using 96-well-based minimal media screening (antibiotic production mapping, APM), pH indicator method, comparative two-dimensional gel electrophoresis (2D-gel), reverse-transcription polymerase chain reaction (RT-PCR), electrophoretic mobility shift assay (EMSA), and scanning electron microscopy (SEM). Using these techniques, we were able to reconstruct a regulatory network describing how these new TRs collectively regulate antibiotic production. This proposed approach providing additional key regulators and their interactions to an existing incomplete regulatory network can also be applied in studying regulators in other bacteria of interest.

Keywords: analytical chemistry; recommendations; sampling; soil; terminology.

INTRODUCTION

The filamentous, soil-dwelling, Gram-positive bacteria *Streptomyces* produce various secondary metabolites, including antibiotics, immunosuppressants, and anticancer agents [1]. For example, the β-lactamase inhibitor, clavulanic acid, is produced by *Streptomyces clavuligerus*, the cancer chemo-

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therapy drug, doxorubicin (DXR), is produced by *Streptomyces peucetius*, and the antiparasitic drug, avermectin, is produced by *Streptomyces avermitilis* [2]. Because of the huge cost of developing new antibiotics and the growing manifestation of multidrug resistance, the antibiotics industry is developing chemically or biologically modified antibiotics as well as hybrid antibiotics, rather than searching for new antibiotics. However, such approaches are usually associated with problems of low product yield. To increase the productivity and yield of antibiotics from recombinant *Streptomyces* strains, various strategies have been applied such as insertion or deletion of global and pathway-specific regulator genes, control of primary metabolism to enhance the concentration of precursors or cofactors, insertion and amplification of biosynthetic genes and clusters for target molecules, replacement of the related promoters, introduction of antibiotics resistance gene, increasing the transport rate of antibiotics by mutation of transporter, and removal of byproducts or branched biosynthetic pathway [3].

After the genome sequencing of *Streptomyces coelicolor* and *S. avermitilis*, it was revealed that the *Streptomyces* strains contained a large number (i.e., 965 for *S. coelicolor*) of regulatory genes [4] compared to other organisms such as *Saccharomyces cerevisiae* (109), *Escherichia coli* (123), and *Bacillus subtilis* (213) [5,6]. This is apparently due to the complex cell metabolism involving cell differentiation and the production of various secondary metabolites.

The effects of many regulators on antibiotic production in *Streptomyces* have been extensively studied [7,8]. For example, overexpression of *actII*-ORF4 and *redD* from *S. coelicolor*, *tylS* and *tylR* from *S. fradiae*, *brpA* from *S. hygroscopicus*, *pikD* from *S. venezuelae*, and *dnrI* from *S. peucetius* increased antibiotic production [9–13]. By contrast, negative regulators of antibiotic biosynthesis such as *aveI* from *S. avermitilis* and *wblA* from *S. coelicolor* decreased the productivity [14,15]. These studies suggested that screening and characterization of new regulators can serve as an effective way to improve the antibiotic productivity. Furthermore, the advances in genomics and proteomics technologies [i.e., DNA sequence analysis, DNA chip technology, two-dimensional gel electrophoresis (2D-gel), chromatin immunoprecipitation with microarray technology (ChIP-Chip), and non-gel-based mass spectrometric protein profiling technologies] that can be used in screening new regulators rapidly improved the procedures of developing industrial strains to increase the antibiotic productivity. In addition, an improved DNA affinity capture assay (DACA) method and clustering analysis of a comprehensive set of DNA chip data also contributed to identifying novel transcriptional regulators (TRs) involved in antibiotic production in *Streptomyces* [16,17].

It is not uncommon for TRs to form a multilayer hierarchical structure, which makes decoding the unique functions of the screened putative TRs challenging [18]. Therefore, we developed a comprehensive framework where a minimal media selection method, 2D-gel, electrophoretic mobility shift assay (EMSA), and scanning electron microscopy (SEM) analysis were integrated to reveal the function of the candidate regulators. Figure 1 is a schematic of our strategic approach to identify new TRs and to characterize their regulatory roles in antibiotic production.

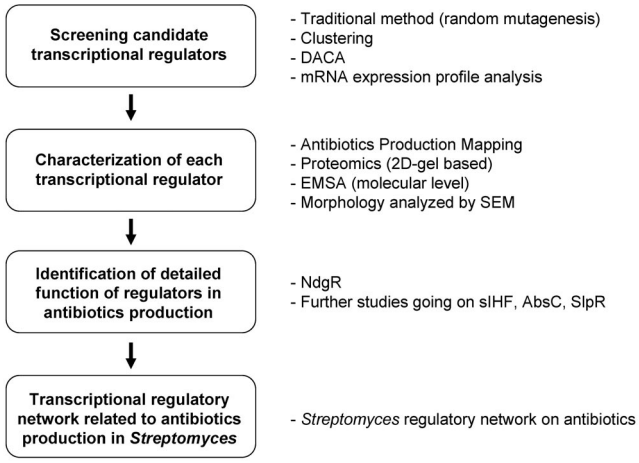

Fig. 1 Strategies to screen and characterize regulators of antibiotic production.

SCREENING OF TRANSCRIPTIONAL REGULATORS—DACA

Although the DACA method developed with mass spectrometric techniques was previously introduced for the screening of DNA-binding proteins, it has not been widely employed because of the long optimization experiments required for efficient screening of TRs [19]. In our previous report [20], an improved DACA combined with mass spectrometry was proposed for the identification of DNA-binding TRs in bacteria. This method includes the following steps as briefly described in Fig. 2. The promoter regions of antibiotic biosynthetic gene clusters were amplified by polymerase chain reaction (PCR)

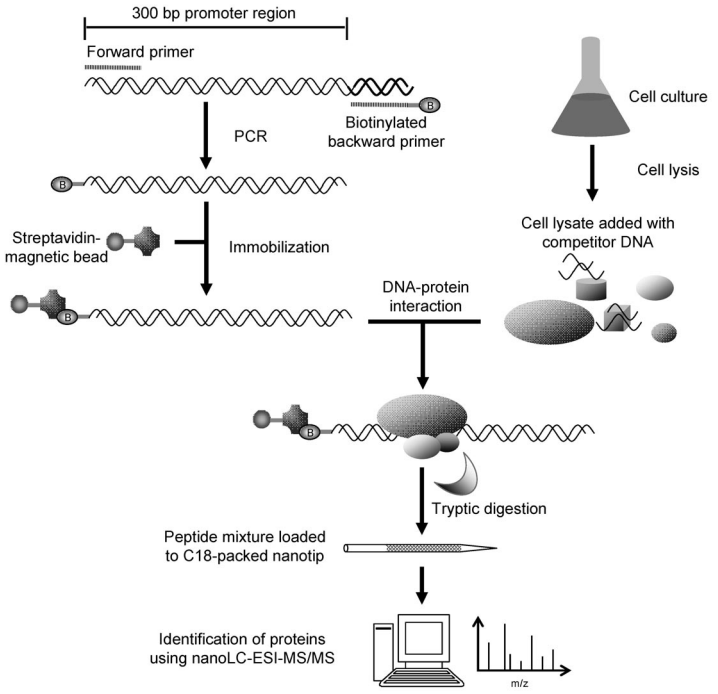

Fig. 2 Schematic procedure of DACA.

using a biotinylated 3'-end primer and its pair. The gel-purified PCR products were then incubated for 30 min with Streptavidin Dynabeads (Dynal Biotech, Oslo, Norway). In parallel, cell lysate was obtained by sonication in lysis buffer containing 500 mM NaCl. To avoid nonspecific binding, sheared salmon sperm competitor DNA was pre-incubated with the cell lysate for 40 min. Next, the bead solution was incubated with the cell lysate at room temperature for 2 h. The beads were then washed and resuspended in ammonium bicarbonate buffer (pH 7.8). Finally, the protein mixture captured on the beads was digested with trypsin and analyzed using an electrospray ionization (ESI)-mass spectrometer (Thermo Scientific, USA).

In our previous study [21], this DACA method was applied to the promoter regions of DXR biosynthetic genes in *S. peucetius.* The results showed that IclR-family TRs, called NdgR (regulator for nitrogen source-dependent growth and antibiotic production), acted as a key regulator for antibiotic production [21], demonstrating the capability of the DACA to effectively find novel candidate regulators controlling any promoter of interest in *Streptomyces*. The same DACA screening method was also applied to the promoter regions of the cluster-specific regulators of actinorhodin (ACT) and undecylprodigiosin (RED) biosynthesis in *S. coelicolor* [16]. The DACA results are summarized in Table 1. Based on the results, the deletion mutants of these candidate regulators were constructed, and their potential functions in antibiotic production are currently being investigated. Although the detailed characterizations of these regulators are not yet completed, they seem to affect the ACT and RED biosynthesis [16,17], suggesting that the optimized rapid screening method is quite efficient and useful to understand the antibiotic production-associated transcriptional networks in *Streptomyces*.

Table 1 DACA results using the promoter regions of *actII-ORF4* and *redD* from *S. coelicolor* (modified from [16]).

$P_{actII\text{-}ORF4}$	
SCO3932	Putative GntR-family transcriptional regulator
SCO0310	Putative TetR-family transcriptional regulator
SCO2792 (AdpA)	Putative AraC-family transcriptional regulator
SCO5405 (AbsC)	Putative MarR-family transcriptional regulator
P_{redD}	
SCO2792 (AdpA)	Putative AraC-family transcriptional regulator
SCO5405 (AbsC)	Putative MarR-family transcriptional regulator
SCO6008 (ROK7B7)	Putative Transcriptional repressor
SCO1480 (sIHF)	Conserved hypothetical protein
SCO3606	Putative regulator
SCO3859	Putative DNA binding protein

IN SILICO SCREENING OF TRANSCRIPTIONAL REGULATORS BASED ON CLUSTERING ANALYSIS OF COMPREHENSIVE DNA CHIP DATASETS

Microarrays have been widely used to understand the global function of target genes of interest. In *S. coelicolor*, there are several DNA chip datasets publicly available for the wild-type or deletion mutants cultured in different conditions at the gene expression omnibus (GEO) [22,23]. In this study, these microarray datasets were used to screen putative TRs based on the assumption that if a regulator is pathway-specific for antibiotic production, the regulator is likely to show mRNA expression patterns similar to antibiotic production.

Therefore, our first approach was to identify putative regulators as the ones showing similar mRNA expression profiles to those of the gene clusters involved in antibiotic production (called antibiotic gene clusters) by clustering a set of DNA microarray data that contain the measurements of mRNA abundance over nine time points during the course of growth of *S. coelicolor* wild-type M145 cultured in $R5^{-}$ liquid media (GSE2983) [24]. Considering that the antibiotic gene clusters showed steep changes in mRNA levels from the exponential to stationary phases, the regulators controlling antibiotic gene clusters are also likely to follow the same dramatic changes producing the large standard deviation (SD) during the transition to stationary phases. We used the SD value of 0.49 as a cut-off value to select TRs in *S. coelicolor* (e.g. *redD*, *actII-ORF4*, *cdaR*, *ramR*, and *absA2*) that might be specifically involved in antibiotic production. Note that the cut-off was determined as the minimum of SD values of the 20 known regulators involved in antibiotic productions. In this way, the global regulators that are constitutively expressed and not likely to be involved in antibiotic production could be removed due to their low SDs. As a result, 51 candidate regulators were generated using this method. To evaluate the potential contributions of these 51 regulators to antibiotic production, a hierarchical clustering was carried out using "unweighted pair group average" as the linkage method and 1—Pearson correlation coefficient as the dissimilarity measure between the temporal expression levels of the 51 regulators and either of the mRNA expression levels of RED gene cluster (SCO5878-SCO5882) or ACT gene cluster (SCO5076-SCO5081, except SCO5079) [25]. The whole procedure was summarized in Fig. 3.

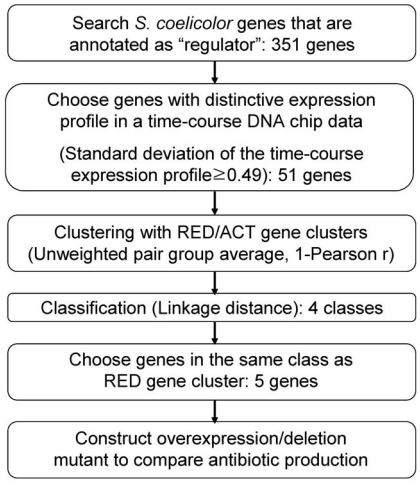

Fig. 3 Flow chart screening TRs involved in ACT/RED production using a DNA chip dataset.

The clustering results allowed us to identify the five regulators, including two known regulators, SCO3579 (WblA) and SCO6722 (SsgD), that had expression patterns close to those of RED biosynthetic genes. SCO3579 (WblA) is known to be an ACT repressor in *S. coelicolor* and on DXR in *S. peucetius* [15]. SCO6722 (SsgD) known to be involved in the synthesis of peptidoglycan along the lateral cell wall synthesis and sporulation might also contribute to antibiotic production [26]. Regarding the other three candidate regulators (SCO1699, SCO0608, and SCO6808), we characterized their potential functions as repressors in antibiotic production by constructing individual overexpression and deletion mutants [17]. It is known that the genes whose products are necessary only for specific period of time during the growth usually act as repressor regulators [27]. Applying this rule to antibiotic production in *Streptomyces*, the fact that most of the identified regulators turned out to be negative regula-

tors was not surprising because antibiotic production is not a highly demanding process occurring only in secondary metabolic phase.

Although this clustering-based approach is useful, it rather generated a list of potential regulator candidates in a nonsupervised fashion when evaluating the similarity between the expression patterns of those regulator candidates and the antibiotic gene clusters. Thus, we developed a more direct approach where we identified putative regulators as the ones whose expression patterns are highly correlated with those of antibiotic biosynthetic gene clusters using more comprehensive microarray datasets than those used in the clustering-based approach. The integration of comprehensive expression profiles from various conditions would allow us to identify a more reliable set of genes than those obtained in the previous approach. Eleven mRNA expression datasets [24,28–31] from the GEO were collected, which include the wild-type growth stage responsive mRNA expression profile [28], the time-course expression profile after various environmental stresses (e.g., osmotic shock, temperature shock, upshift of phosphate concentration in the culture media) or the time-course expression profile of deletion mutants (e.g., regulators such as *afsS*, *absB*, and *absA1*) [28–31].

After combining the raw intensities at gene level from each dataset, the intensities were normalized using quantile normalization [32]. Then, log2 ratios of the intensities from each time point to those at $t = 0$ (control) were calculated as fold changes in each dataset. The genes whose fold change patterns were highly correlated with those of antibiotic clusters such as RED and ACT were then identified using an integrated statistical hypothesis testing as follows: First, for each gene, two p-values were computed from the two hypothesis tests based on Pearson correlation and Spearman rank correlation, respectively, using the fold change profiles across all conditions. Second, the overall p-value was then estimated using Stouffer's method [33] by combining the two individual p-values above. Finally, we selected the potential regulators with an overall p-value less than 1e-50 and also with a maximum log2 ratio higher than 0.58 across all conditions: 119 and 85 genes were selected using RED and ACT clusters, respectively. Since we were primarily interested in regulators, only the genes that have been annotated as putative regulators were selected from the 119 and 85 genes, resulting in 12 and 5 putative regulators, respectively.

In these putative regulators, some genes were already reported to affect antibiotic production. Interestingly, SCO0608 and SCO1699 that were found as potential regulators of antibiotic production from the clustering-based method and then shown they function as negative regulators [17] were categorized once again to have the same mRNA profile as the RED gene cluster in our selected genes. Among the others, *sIHF* (SCO1480) had the same mRNA profile as the RED gene cluster, which overlapped with our targets screened by DACA and by clustering one set of DNA chip data as discussed above. Thus, these observations are highly consistent with previous findings, thus collectively supporting the validity of our methods in identifying potential regulators of antibiotic production in *Streptomyces*.

INITIAL METHODS FOR THE CHARACTERIZATION OF TRANSCRIPTIONAL REGULATORS: APM, pH INDICATOR METHOD, 2D-GEL

The functions of 17 candidate regulators we have screened have not been characterized yet and thus annotated as "hypothetical" or "putative" regulators. Although these can be classified into a specific family such as tetracycline transcriptional regulator (TetR) or multiple antibiotic resistance transcriptional regulator (MarR), there are many regulators with the same family name. In the *Streptomyces* database (<http://strepdb.streptomyces.org.uk>), there are 185 TetR family regulators and 42 MarR family regulators. Accordingly, this simple annotation based upon the homology of protein sequences is not sufficient to identify their functional roles.

In microbial physiology, phenotype comparison with deletion mutants and cell growth measurement in minimal media are a well-known technique to identify unknown functions of target genes. To unravel the functions of a new TR, it is important to screen various media components (carbon and

nitrogen sources) and to design optimal media compositions that yield different morphology and/or antibiotic production compared to the wild type. Furthermore, *S. coelicolor* growth conditions and production of antibiotics such as blue pigment ACT and red pigment RED are greatly affected by nutrient composition [7,34], and, in minimal media, it is easier to observe the small differences in cell growth and metabolism between the wild-type and knock-out mutants. To have some clues on the functions of the TRs whose functions are not characterized, antibiotic production mapping (APM) technique was developed using 96 well plates containing minimal media with the combination of 12 different carbon sources and 16 amino acids as nitrogen sources (192 combinations). The colors of the cultured cells of the wild-type and the deletion mutant were compared. This method is more useful for the strains producing color pigments such as *Streptomyces* species.

In the case of NdgR whose sequence shows a high similarity among the *Streptomyces* strains, the deletion mutant of SCO5552 was constructed in *S. coelicolor* [21]. BG11 (*S. coelicolor* Δ*ndgR*) produced more ACT than the wild type (M145) in the minimal media containing the amino acids including leucine, glutamine, asparagine, and isoleucine, and various carbon sources via the APM method [21]. This method saves both efforts and time by allowing us to roughly identify the function of the newly found TRs. Thus, it is useful in characterizing the function of unknown regulators (Fig. 4).

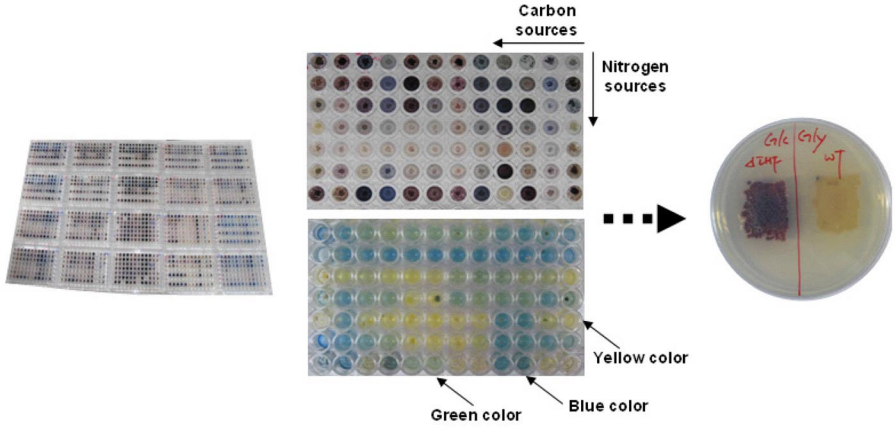

Fig. 4 Example of the 96-well plate showing differential antibiotic production in various carbon and nitrogen sources. pH changes were detected in different colors due to bromophenol blue, and its application to the selection of minimal media that have distinct antibiotic production pattern in the deletion mutant compared to the wild type (Δ*sIHF*).

In many cases, the deletion of one regulator may affect the balance of tricarboxylic acid (TCA) cycle or glycolysis, and the imbalance between the two pathways might alter the secretion of organic acids such as pyruvate, acetate and α-ketoglutarate [35]. Nutrient-dependent pH changes in the minimal media can provide information regarding the occurrence of such an imbalance, when the pH changes were monitored using pH indicator bromothymol blue, thus permitting us to understand which nutrient combinations caused a pH-shift. Bromothymol blue is a chemical indicator that changes color from green to yellow in acidic solutions and to blue in basic solutions. Basically, the amount of various organic acids produced from the TCA cycle starting from acetyl-CoA was reduced when the carbon flux was channeled to polyketide antibiotic synthesis starting from acetyl-CoA and malonyl-CoA. Therefore, the pH change is correlated with and the antibiotic production and provides valuable information on the link between the primary and secondary metabolisms of the cell [36].

To elucidate the functions of novel regulators that have complex roles in detail, 2D-gel followed by nanoLC-LTQ orbitrap mass spectrometry was used. This proteomic approach can present a global

view of differential protein expression between wild-type and deletion mutants. In addition, this method can also be used as an initial tool characterizing unknown regulators. For example, the proteome of *S. coelicolor* BG11 (Δ*ndgR*) was compared with that of the wild-type strain [21]. Analysis of the protein spots that were up/down-regulated more than two-fold showed the increase in abundances of ACT biosynthetic enzymes and antioxidant enzymes and the decrease in abundances of molecular chaperones and several proteins involved in energy generation, indicating the delayed growth of BG11. In addition, the decreased expression of a branched-chain amino acid aminotransferase (IlvE) was in agreement with the APM results where ACT production was enhanced in minimal media containing branched-chain amino acids such as leucine, isoleucine, and valine [37]. The overall changes in protein expression were due to both direct and indirect regulations on both primary and secondary metabolism mediated by NdgR.

DETAILED CHARACTERIZATION OF TRANSCRIPTIONAL REGULATORS USING RT-PCR, EMSA, SEM, AND ITS APPLICATION TO NdgR

Target genes identified by proteomic tools may be further investigated to understand their mode of interaction with the regulatory protein by either reverse-transcription PCR (RT-PCR) or EMSA. For example, the effects of NdgR on the transcriptional activity of the genes involved in antibiotics and amino acid syntheses were identified by comparing the mRNA expression levels of glutamine, leucine, RED, and ACT biosynthetic genes by RT-PCR [20]. Decreased expression levels of *leuC* and *leuD* correlated with the increased ACT production in leucine minimal media. Decreased expression of the genes in the RED cluster (*redD*, *redZ*, and *redQ*) and slight increased expression of the genes in the ACT cluster (*actII-ORF4*, *actIVA2*, and *actIVA4*) were observed in BG11.

Moreover, EMSA allowed us to examine whether or not NdgR could directly regulate the genes involved in amino acid metabolism and antibiotic production by binding to their promoters. NdgR protein was overexpressed and purified as His-tagged protein in *E. coli*. Binding of NdgR to the promoters of *glnA* and *glnII* encoding glutamine synthetase A and glutamine synthetase II, respectively, and promoters of *redD* and *actII-ORF4* were tested [20]. Surprisingly, NdgR did not bind to any of these promoters, suggesting that NdgR's effect on glutamine metabolism and antibiotic production appear to be indirect. In addition, the SEM image of BG11 presented shorter aerial mycelia and defects in cell differentiation when cultured on minimal media agar plate with glutamine as a nitrogen source.

CONCLUSION

Several unknown putative TRs binding on the promoter regions of antibiotic biosynthesis gene clusters such as ACT and RED were identified using a modified DACA method and two statistical clustering methods developed in our group [21]. As a model system, NdgR (SCO5552) was demonstrated as an example throughout this paper, but most of the screened TRs were indeed DNA-binding proteins and affected antibiotic production to a certain degree (data not shown). To further identify the exact functions of the TRs and their mechanisms of action, various genomics and proteomics tools such as DNA chip, EMSA, 2D-gel analysis, and RT-PCR were used with a 96-well minimal media culture assay (i.e., APM) with deletion mutants. The identification process of the TR functions is undergoing, and the characterization of the TRs and their roles will be published in the near future.

As the experimental data on the deletion mutants of TR genes have been collected, the direct/indirect association of such TRs with antibiotic production as well as their complex regulatory networks among TRs has drawn our attention. For that reason, we have attempted to reconstruct a basic network connecting *Streptomyces* TRs using the Cytoscape 2.6.0 program based on our experimental data combined with published data (Fig. 5). This basic network could help us gain deeper insights into how TRs interact and contribute to antibiotic synthesis in *S. coelicolor* under different nutrient conditions. Importantly, the result can also be used to decide what combinations of regulators should be selected

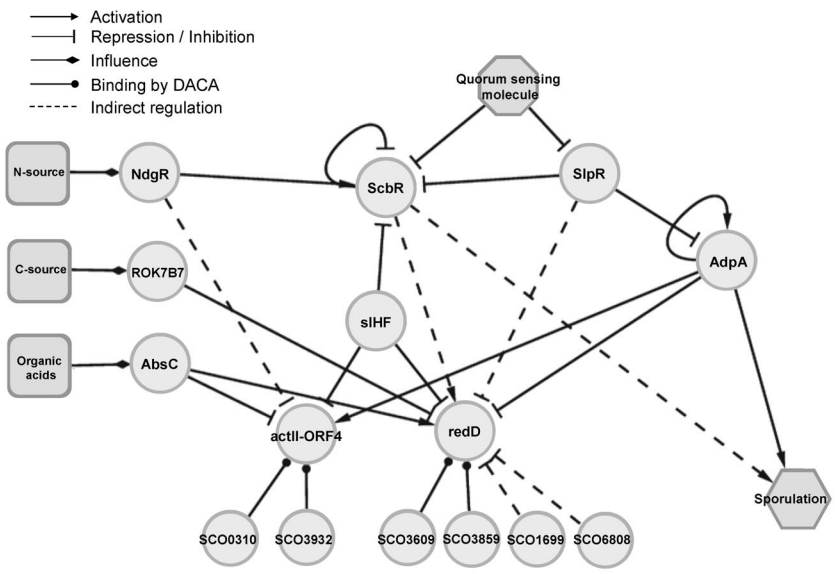

Fig. 5 TR network of antibiotic production in *S. coelicolor* based on our experimental data.

for the maximal production of antibiotics leading to the effective construction of industrially useful strains in the future after further integration of additional quantitative information (e.g., degree of contributions of the individual regulators to antibiotic production) into the network. Furthermore, our strategy to find new TRs in a high-throughput manner can also be applied to other bacteria that have little additional information besides the genomic sequence.

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