1 **Sulfane sulfur post-translationally modifies the global regulator AdpA to**
2 **influence actinorhodin production and morphological differentiation of**
3 ***Streptomyces coelicolor***

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23 ABSTRACT

24 The transcription factor AdpA is a key regulator controlling both secondary
25 metabolism and morphological differentiation in *Streptomyces*. Due to its critical
26 functions, its expression undergoes multi-level regulations at transcriptional,
27 post-transcriptional, and translational levels, yet no post-translational regulation has
28 been reported. Sulfane sulfur, such as organic polysulfide (RS_nH , $n \geq 2$), is common
29 inside microorganisms, but its physiological functions are largely unknown. Herein,
30 we discovered that sulfane sulfur post-translationally modifies AdpA in *S. coelicolor*
31 via specifically reacting with Cys⁶² of AdpA to form a persulfide (Cys⁶²-SSH). This
32 modification decreases the affinity of AdpA to its self-promoter P_{adpA} , allowing
33 increased expression of *adpA*, further promoting the expression of its target genes
34 *actII-4* and *wblA*. ActII-4 activates actinorhodin biosynthesis and WblA regulates
35 morphological development. Bioinformatics analyses indicated that AdpA-Cys⁶² is
36 highly conserved in *Streptomyces*, suggesting the prevalence of such modification in
37 this genus. Thus, our study unveils a new type of regulation on the AdpA activity and
38 sheds a light on how sulfane sulfur stimulates the production of antibiotics in
39 *Streptomyces*.

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45 **IMPORTANCE**

46 *Streptomyces* produce myriad of polyketide compounds having (potential) clinical
 47 applications. While the database of polyketide gene clusters are quickly expanding,
 48 the regulation mechanisms of them are rarely known. Sulfane sulfur species are
 49 commonly present in microorganisms with unclear functions. Herein, we discovered
 50 that sulfane sulfur increases actinorhodin (ACT) production in *S. coelicolor*. The
 51 underlying mechanism is sulfane sulfur specifically reacts with AdpA, a global
 52 transcription factor controlling both ACT gene cluster and morphological
 53 differentiation related genes, to form sulphydrated AdpA. This modification changes
 54 dynamics of AdpA-controlled gene network and leads to high expression of ACT
 55 biosynthetic genes. Given the wide prevalence of AdpA and sulfane sulfur in
 56 *Streptomyces*, this mechanism may represent a common regulating pattern of
 57 polyketide gene clusters. Thus, this finding provides a new strategy for mining and
 58 activating valuable polyketide gene clusters.

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61 **INTRODUCTION**

62 *Streptomyces* spp. are gram positive bacteria with a filamentous form, which
 63 colonize a wide range of terrestrial and aquatic niches. The most famous characteristic
 64 of *Streptomyces* is the ability to produce myriad of secondary metabolites including
 65 antibiotics, antifungal, antiviral, anthelmintic agents, antitumoral drugs,
 66 anti-hypertensives, herbicides, and interesting pigments (1-3). Numerous efforts have

67 been spent on searching, identifying, and modifying gene clusters responsible for
68 biosynthesis of these secondary metabolites (4). In contrast, much less energy has
69 been invested in illustrating the transcriptional/translational regulation of these gene
70 clusters. One reason is *Streptomyces* have a complex life cycle that includes
71 sporulation, a vegetative or substrate state, and aerial mycelial growth. The
72 biosynthesis of secondary metabolites is closely linked to the stages of life cycle (5, 6),
73 which makes relative studies challengeable.

74 AdpA is a transcriptional regulator universally present in *Streptomyces* (7). It
75 locates in the second layer of the A-factor-dependent transcriptional network in
76 *Streptomyces griseus*, the first layer is the A-factor receptor, which activates AdpA
77 expression at the present of A-factor (γ -butyrolactone, a quorum sensing hormone).
78 Therefore, AdpA expression is indirectly controlled by the quorum sensing signal.
79 Aside from A-factor, there are at least three players in AdpA expression regulation,
80 including the master developmental regulator BldD regulating at transcriptional level
81 (8, 9), a *cis*-antisense RNA regulating at post-transcriptional level (10), and a rare
82 tRNA (tRNA^{UUA}_{L_{eu}}) encoding gene *bldA* regulating at translational level (11). It was
83 also reported that AdpA can be transcriptionally self-inhibited (12). Why regulation of
84 AdpA expression is such multiple and complicated might be owing to that AdpA is a
85 key regulator of both secondary metabolism and morphological differentiation (13).
86 Considering the critical functions it conducts, whether there are other players
87 regulating at different levels of AdpA expression or activity is unclear but worthy of
88 further investigation.

89 Sulfane sulfur containing compounds, such as persulfide (HSSH and RSSH) and
 90 polysulfide (HSS_nH, S_n, RSS_nH, RSS_nR, n≥2), are commonly present in both
 91 eukaryotic and prokaryotic cells (14). In the latest two decades, intensive studies of
 92 sulfane sulfur have been performed with mammalian cells because it is found that
 93 sulfane sulfur is involved in the regulation of diverse physiological and pathological
 94 processes including apoptosis, carcinogenesis, and redox maintenance (15-18). On the
 95 other hand, studies of microorganism sulfane sulfur are traditionally focused on its
 96 metabolism and its role in the global sulfur cycle (19, 20). In recent years, the
 97 physiological functions of sulfane sulfur in microorganisms also attract attentions. For
 98 instance, Peng et al. (21) found that sulfane sulfur regulates the expression of
 99 virulence factors in *Staphylococcus aureus*, Liu et al (22) reported that sulfane sulfur
 100 is involved in photosynthesis regulation in *Synechococcus*. Albeit being noticed, the
 101 functions of sulfane sulfur in microorganisms are largely unveiled.

102 In a previous study, we discovered that sulfane sulfur functions as a signal to
 103 activate actinorhodin (ACT) production in *S. coelicolor* M145, a model strain of
 104 *Streptomyces*. In addition, the spore formation process is accelerated by endogenously
 105 accumulated sulfane sulfur (23). These phenomena suggest that sulfane sulfur affects
 106 both secondary metabolism and cell cycle in *S. coelicolor* M145. Based on these
 107 findings, we herein studied the underlying mechanism of how sulfane sulfur perform
 108 such functions. We found that AdpA is the key media of sulfane sulfur signaling.
 109 AdpA senses the level of intracellular sulfane sulfur and adjusts ACT production and
 110 spore formation. Even the expression of AdpA itself is affected by sulfane sulfur, i.e.

111 sulfane sulfur is a new regulator of AdpA. Thus, this study unveils one way of how
112 sulfane sulfur signals in *Streptomyces*.

113

114 **RESULTS**

115 **AdpA is a key regulator of ACT production and morphological development in *S.***

116 *coelicolor*. Previous studies reported that AdpA involves in the regulation of ACT
117 production and morphological development in *S. coelicolor* (24, 25). Herein, we
118 constructed an *adpA*-disrupted *S. coelicolor* M145 strain ($\Delta adpA$). It exhibited a
119 phenotype of no ACT but high undecylprodigiosin (RED) production when cultured
120 on YBP agar medium (Fig. 1A). Complementary expression of *adpA* gene using a
121 plasmid pMS82-*adpA* ($\Delta adpA::adpA$) restored ACT production, while the control,
122 $\Delta adpA$ harboring an empty plasmid ($\Delta adpA::pMS82$), showed no change. In addition,
123 we noticed that both $\Delta adpA$ and the control showed a bald and non-spore form on
124 YBP medium (Fig. 1A) while $\Delta adpA::adpA$ restored the spore formation. These
125 results verified that AdpA controls ACT production and morphological development
126 in *S. coelicolor* M145 strain.

127

128 **Sulfane sulfur performing ACT activation effect requires the presence of AdpA.**

129 Since the $\Delta adpA$ strain displayed opposite phenotypes as that of sulfane sulfur treated
130 strain (11), we suspected that AdpA had interwound functions with sulfane sulfur. We
131 performed sulfane sulfur induction experiments using the *S. coelicolor* M145 (wt),
132 $\Delta adpA$ and $\Delta adpA::adpA$ strains. The strains were spread on YBP medium containing

133 1 mM thiosulfate or 0.1 mM cysteine (which can be converted to sulfane sulfur *in*
134 *vivo*) and cultured at 30°C for 10 days. For wt, the production of ACT was
135 significantly increased by thiosulfate/cysteine treatment (Fig. 1B and 1C). For $\Delta adpA$,
136 no production of ACT was observed with or without thiosulfate/cysteine treatment.
137 For $\Delta adpA::adpA$, the induction effects were similar as in wt (Fig. 1B and 1C). These
138 results indicated that AdpA is required for sulfane sulfur to execute the ACT
139 production activating function.

140
141 **Fig. 1. AdpA is required for ACT production and morphological development in**
142 ***S. coelicolor* M145.** (A) Phenotypes of the wt, $\Delta adpA$, $\Delta adpA::adpA$, and
143 $\Delta adpA::pMS82$ strains grown on YBP medium at 30°C. Images were taken at the
144 indicated times. (B) 1 mM thiosulfate or 0.1 mM cysteine were added into YBP agar
145 plates before inoculation. The plates were incubated at 30°C for 10 days, images were

146 captured from the reverse side of the plates. (C) Quantitative determination of ACT
147 produced by the wt, $\Delta adpA$ and $\Delta adpA::adpA$ strains on YBP containing thiosulfate or
148 cysteine, the plates were incubated at 30°C for 10 days and then subjected to analysis.
149 Data were from three independent repeats. (D-E) wt and $\Delta adpA$ strains were grown on
150 YBP liquid medium, when cultured for 36 h, 400 μ M HS_nH or S₈ were added. After 1
151 h, RNA samples were isolated after induction. The y-axis shows the fold change of
152 *actII-4* expression levels in wt and $\Delta adpA$. Real-time PCR data were from three
153 independent repeats and shown as average $\pm$ SD.

154

155 ActII-4 is the ACT production “pathway-specific” activator (25, 26). We analyzed
156 transcription of *actII-4* using the real-time quantitative reverse transcription PCR
157 (RT-qPCR) method. The wt and $\Delta adpA$ strains were treated with two sulfane sulfur
158 containing chemicals, hydrogen polysulfides (HS_nH, n $\geq$ 2) and sublimed sulfur (S₈).
159 For wt, the transcription level of *actII-4* was much higher in treated strain than that in
160 untreated one (Fig. 1D and 1E); whereas for $\Delta adpA$, the transcription level of *actII-4*
161 had no obvious change after sulfane sulfur treatment (Fig. 1D and 1E). These results
162 indicated that sulfane sulfur can increase ActII-4 expression, which subsequently
163 activates ACT production, but this process requires the presence of AdpA.

164

165 Sulfane sulfur affects the interaction between AdpA and its cognate promoters.

166 AdpA controls the transcription of *actII-4* and *wblA* (*whiB* like gene A, which
167 controls morphological development in *S. coelicolor*) via binding to their promoters
168 (27). Using these two promoters and an enhanced-green-fluorescence-protein
169 encoding gene (*egfp*), we constructed two reporter systems (Fig. 2A and 2B). These
170 reporter systems were introduced into the wt and $\Delta adpA$ strains. HS_nH was used to
171 treat the strains containing the reporter systems. After 30 min treatment, the mycelium

was collected by centrifugation and the fluorescence was read by a fluorophotometer.

In the wt strain, HS_nH treatment enhanced the strength of both *actII-4* promoter ($P_{actII-4}$) and *wblA* promoter (P_{wblA}), evidenced by the increased EGFP expression.

Whereas, in the $\Delta adpA$ strain, EGFP expression was not increased but decreased after HS_nH treatment, indicating that HS_nH treatment lost the enhancing effect on these promoters. These results suggested that sulfane sulfur may affect the interaction between AdpA and its cognate promoters.

Fig. 2. Sulfane sulfur is involved in the process of AdpA regulating target genes. (A) HS_nH was used to treat wt and $\Delta adpA$ strains harboring pMS82-*actII-4p-egfp*. (B) HS_nH was used to treat wt and $\Delta adpA$ strains harboring pMS82-*wblAp-egfp*. Data were from three independent repeats and shown as average $\pm$ SD. (C,D) EMSA analysis of the AdpA affinity to $P_{actII-4}$ promoter DNA (C) and the P_{wblA} promoter DNA (D). All lanes contained 20 nM probe DNA, lanes 2-5 contained the indicated

186 protein concentration, lanes 3-5 contained the HS_nH (left) or S₈ (right). Black arrow
187 indicates the free DNA probe and red arrow indicates *P_{actII-4}*-AdpA or *P_{wblA}*-AdpA
188 complex.

189

190 We then performed electrophoretic mobility shift assays (EMSA) to investigate the
191 interaction. The AdpA protein was expressed in *E. coli* BL21 (DE3) and purified. The
192 DNA probes of the *wblA* and *actII-4* promoters were obtained by PCR. When AdpA
193 was mixed with the *P_{actII-4}* or *P_{wblA}* DNA probe, it bound to them (Fig. 2C and 2D).
194 When HS_nH or S₈ was also added, the fraction of the AdpA-probe complexes
195 decreased (Fig. 2C and 2D). These results indicated that sulfane sulfur decreased the
196 affinity of AdpA to *P_{actII-4}* and *P_{wblA}*.

197 To test whether the influence of sulfane sulfur can be reversed by a reductant, we
198 added dithiothreitol (DTT) into the mixture of sulfane sulfur, AdpA, and the *P_{wblA}*
199 probe (the DTT dosage was 2 folds of HS_nH/S₈). After DTT treatment, AdpA restored
200 the high affinity with the *P_{wblA}* probe (Fig. 3), which had been attenuated by sulfane
201 sulfur. These phenomena demonstrated that the affinity attenuation of AdpA to its
202 cognate DNA caused by sulfane sulfur is reversible.

203

205 **Fig. 3. EMSA analysis of AdpA binding to *P_{wblA}* promoter DNA.** All lanes
206 contained 22 nM *P_{wblA}* DNA, lanes 2-7 contained AdpA, lanes 4 and 5 contained

207 HS_nH, lanes 6 and 7 contained S₈, lanes 5 and 7 contained DTT.

208

209 **Sulfane sulfur also affects the transcription of AdpA itself.** The *adpA* gene is
 210 transcriptionally self-controlled (28). There are five AdpA binding sites in the *P_{adpA}*
 211 promoter (Fig. 4A). We designed a pair of primers (*adpA-wt*) from the undeleted part
 212 of *adpA* gene (Fig. 4B) and used these primers to analyze the transcription change of
 213 *adpA* in wt and $\Delta adpA$. The transcription level of undeleted part was ~30-fold higher
 214 in $\Delta adpA$ than that in wt, indicating that in the absence of AdpA, the strength of *P_{adpA}*
 215 is higher, i.e., AdpA acts as a repressor for its own transcription (Fig. 4C)

216 To test whether sulfane sulfur can affect this self-repression, we compared
 217 transcription levels of *adpA* in the wt strain and the Δpdo strain. In the latter
 218 intracellular sulfane sulfur is accumulated due to lack of the persulfide oxidation gene
 219 (*pdo*) (23). Results showed that the *adpA* transcription levels were higher in Δpdo
 220 than in wt (Fig. 4D). We then used exogenous sulfane sulfur to treat the wt strain and
 221 found that both HS_nH and S₈ can increase *adpA* transcription (Fig. 4E and 4F). EMSA
 222 showed that sulfane sulfur also reduced the affinity of AdpA to *P_{adpA}* probe, as the
 223 unbound probe increased after the addition of HS_nH and S₈ (Fig. 5A). Fluorescence
 224 polarization (FP) analysis was performed, and results showed that HS_nH obviously
 225 increased the K_D value (the equilibrium dissociation constant) of AdpA to the *P_{adpA}*
 226 probe, as well as to the *P_{wblA}* probe (Fig. 5B and 5C), indicating that the affinity of
 227 AdpA to these promoters was attenuated by HS_nH. Together, these results indicated
 228 that sulfane sulfur attenuates the self-repression of *adpA* transcription.

Fig. 4. Sulfane sulfur affects the transcription of *adpA* itself. (A) Schematic diagram of the the AdpA binding sites in *adpA* promoter region. (B) Schematic diagram of the the AdpA coding sequence. The fragment covers 169 bp to 1107 bp was deleted in $\Delta adpA$. The *adpA*-wt and *adpA*-de primers were used to test the undeleted and deleted sequences, respectively. (C) RT-qPCR analysis of *adpA*-wt mRNA level in wt and $\Delta adpA$. (D) RT-qPCR analysis of *adpA*-wt mRNA level in wt and Δpdo . The expression level in wt at 36 h was arbitrarily set to one. The y-axis shows the fold change of transcription levels in wt and Δpdo over the levels in wt at 36 h. Data were from three independent repeats and shown as average $\pm$ SD. (E,F) RT-qPCR analysis of *adpA*-wt in wt and $\Delta adpA$ after inducing by HS_nH (E) and S_8 (F) for 1 h. Data were from three independent repeats and shown as average $\pm$ SD.

Fig. 5. EMSA and FP analysis of AdpA binding to DNA probes. (A) EMSA analysis of AdpA binding to P_{adpA} probe. All lanes contained 20 nM probe, lanes 2-13 contained AdpA, lanes 5-7 contained H₂S, lanes 8-10 contained HS_nH, lanes 11-13 contained S₈. (B,C) FP analysis of AdpA binding to P_{adpA} probe (B) and P_{wblA} probe (C). 1 nM FAM-labeled P_{adpA} or P_{wblA} was incubated with increasing amounts of AdpA or HS_nH treated AdpA. The K_D values were calculated using GraphPad Prism 5 software. Data were from three independent experiments and shown as average $\pm$ SD.

Kinetics simulation of the AdpA controlled promoter system. To better understand

how sulfane sulfur influences the performances of $P_{actII-4}$, P_{wblA} , and P_{adpA} , we used

two mathematical models to simulate the dynamics of the promoter system. For

simplification, we only took account of the influence of AdpA and sulfane sulfur on

the system (Fig. 6). Equations and related parameters used for modelling are provided

in supplementary materials. When cellular sulfane sulfur is low, P_{adpA} and AdpA

compose a classic closed negative-feedback loop. Therefore, the P_{adpA} strength

displays an oscillating dynamics, leading to oscillating AdpA expression. Since the

expression of ActII-4 and WblA is activated by AdpA, their expression oscillates correspondingly with a short delay (Fig. 6A). When cellular sulfane sulfur is high, it attenuates the AdpA repression on P_{adpA} and breaks the negative-feedback loop. When the closed loop is totally broken, P_{adpA} is constantly on, leading to a constant level of AdpA and resulting in constant expression of ActII-4 and WblA (Fig. 6B). The variation of cellular sulfane sulfur between the low and high levels will affect the expression of *adpA*, *actII-4*, and *wblA*.

267

Fig. 6. Modelling dynamics of the AdpA controlled promoters. (A) In the absence of sulfane sulfur, P_{adpA} is self-repressed by AdpA to form a negative-feedback loop, and hence both P_{adpA} strength and AdpA amount show an oscillating dynamics. (B) Sulfane sulfur breaks the negative-feedback loop, which leads to a constant expression of AdpA, ActII-4, and WblA. Equations and related parameters used for modeling are provided in supplementary materials.

274

The cysteine residue Cys⁶² is critical for AdpA sensing sulfane sulfur. Sulfane

sulfur can react with cysteine residues of certain proteins to change their

configurations (29, 30). AdpA contains four cysteine residues, Cys⁶², Cys¹²⁶, Cys¹⁸⁷,

and Cys³⁰⁷. To find out which cysteine residue involves in the AdpA-sulfane sulfur

279 interaction, we made cysteine-to-serine mutation on each cysteine residue of AdpA.

280 The mutated *adpA* genes were complemented in the $\Delta adpA$ strain. When growing in

281 YBP agar medium, the $\Delta adpA::adpA_{C126S}$, $\Delta adpA::adpA_{C187S}$, and $\Delta adpA::adpA_{C307S}$

282 strains did not show obvious difference from the wt and $\Delta adpA::adpA$ strains.

283 However, the $\Delta adpA::adpA_{C62S}$ strain was distinct from others. It lost the ability of

284 generating spores and its ACT production was also apparently lower (Fig. 7A). These

285 phenotype changes indicated that Cys⁶² is critical for AdpA performing its regulatory

286 function.

287

288

289 **Fig. 7. Cys⁶² residue is critical for AdpA sensing sulfane sulfur.** (A) Phenotypes of

290 wt, $\Delta adpA$ and complementary strains ($\Delta adpA::adpA_{C62S}$, $\Delta adpA::adpA_{C126S}$,

291 $\Delta adpA::adpA_{C187S}$ and $\Delta adpA::adpA_{C307S}$) grown on YBP medium. Images were

292 captured from both sides of the plates. (B) EMSA analysis of AdpA_{C62S} binding to

293 P_{wblA} DNA. All lanes contained 16.5 nM probe DNA, lanes 2-10 contained AdpA,

294 lanes 5-7 contained HS_nH and lanes 8-10 contained S₈. Black arrow indicates the free

295 DNA probe, and red arrow indicates P_{wblA} -AdpA_{C62S} complex.

296

297 EMSA was then performed to examine how C62S mutation affected the binding of
298 AdpA to its cognate promoter. AdpA_{C62S} still bound to *P_{wblA}* DNA fragment, but the
299 binding was no longer influenced by sulfane sulfur even when sulfane sulfur was
300 added at high concentrations (>1000 fold higher than that of AdpA_{C62S}) (Fig. 7B).

301 To check how sulfane sulfur reacts with AdpA, purified AdpA was treated with
302 HS_nH or DTT. The treated-AdpA was labeled with iodoacetamide (IAM) and then
303 subjected to trypsin digestion, followed by LTQ-Orbitrap tandem mass spectrometry
304 analysis. For the HS_nH treated-AdpA, two peptides (1 and 2, Fig. 8) were identified.
305 In peptide 1 (1299.67 Da), the Cys⁶² residue was directly blocked by IAM to form
306 Cys⁶²-AM (acetamide) (Fig. 8 and Fig. S1). In peptide 2 (1331.64 Da), a mass
307 increase of 32 (+32 MW) was identified. MS² spectrum indicated that the +32 MW
308 happened on thiol group of Cys⁶² to form peptide-S-AM (Fig. 8 and Fig. S2). The
309 MS¹ signal intensity ratio of peptide 1/peptide 2 was 17 %, As the control, only a
310 peptide with Cys⁶²-AM (1299.67, peptide 3) was identified, corresponding to a direct
311 blockage of IAM on Cys⁶² residue (Fig. 8 and Fig. S3). These results indicated that
312 sulfane sulfur can modify Cys⁶²-SH to form Cys⁶²-SSH.

313

Fig. 8. LC-MS/MS analysis of HS_nH treated and DTT treated AdpA.

MS² data of the peptides are provided in Figs S1–S3.

The thiol group of Cys⁶² is accessible to solution due to its location on the AdpA

3D structure. The 3D structure of AdpA was modelled by using AlphaFold 2

(<https://cryonet.ai/af2/>). The crystal structure of a truncated AdpAsg containing only

the DNA binding domain, which is from *Streptomyces griseus*, is available in PDB

database (PDB: 3w6v). We aligned AdpAsg with the modelled AdpA, and the

alignment parameter RMSD was 0.365, indicating a high confidence of the predicted

structure of AdpA (Fig. 9A and 9B).

We then analyzed locations of the four cysteine residues in AdpA (Fig. 9C). Cys⁶²,

Cys¹²⁶, and Cys¹⁸⁷ locate in the ThiJ/PfpI/DJ-1-like dimerization domain and Cys³⁰⁷

locates in the AraC/XylS-type DNA-binding domain (DBD). Cys¹⁸⁷ and Cys³⁰⁷ fold

into interior of AdpA, and hence they are protected from sulfane sulfur attack.

Differently, Cys⁶² locates near the protein surface, and its thiol group is exposed to

solution, which may explain why Cys⁶² can be modified by sulfane sulfur. It is

noteworthy that Cys¹²⁶ locates on the interior surface of a tunnel through the

dimerization domain. Therefore, sulfane sulfur compounds may not enter this tunnel

333 to react with its thiol group. In addition, the distance between any two cysteine
334 residues is too far to form a disulfide (S-S) or tri-sulfide (S-S-S) bond.

335
336 **Fig. 9. AlphaFold 2-predicted 3D structure of AdpA.** (A, B) Alignment of the
337 predicted AdpA structure (multicolor) with the AdpAsg crystal structure (grey). (C)
338 Locations of the cysteine residues in AdpA. Yellow spheres represent the sulfur
339 atoms.

340

341 **The cysteine residues are conserved in *Streptomyces* AdpAs.** We analyzed AdpA
342 and its homologues in *Streptomyces* genus. In the PATRIC database, 2752 of 3033
343 sequenced *Streptomyces* strains contain AdpA, accounting for a 90.73% prevalence.
344 We selected some representative AdpA sequences to construct a phylogenetic tree.

Results revealed that AdpA homologues were not on one evolutionary branch (Fig. S4). However, when we performed multiple sequence comparison with them, we found that their four cysteine residues were highly conserved, including Cys⁶² (Fig. S5). These results suggested that using cysteine residues to sense sulfane sulfur may be a common mechanism for AdpA functioning in *Streptomyces*.

350

351 DISCUSSION

The global transcription factor AdpA plays an important role in regulation of secondary metabolism and morphological development in the *Streptomyces* genus (31-35). Its own expression is controlled by multiple factors. In this study, we discovered that sulfane sulfur affects AdpA activity via the post-translational modification in *S. coelicolor* M145. After reacting with sulfane sulfur, the affinity of AdpA to its cognate promoters, P_{adpA} , $P_{actII-4}$, and P_{wblA} , is attenuated, which increases the expression of AdpA, *actII-4* and *wblA*. We constructed a simplified model to understand the effect of sulfane sulfur on the AdpA-controlled promoters. As shown in our simulation (Fig. 6), P_{adpA} is under the control of a negative feedback loop of self-repression. Without presence of sulfane sulfur and/or other disturbing factors, activation of AdpA on *actII-4* and *wblA* expression cannot last long time due to the feedback. Sulfane sulfur modifies AdpA to break/remove the self-repression, and then the activation can last longer. Thus, the effect of sulfane sulfur on the AdpA regulon may represent a fine-tuned regulation for the production of antibiotics and morphological development. Further investigation is required to understand the fine

367 regulations.

368 Furthermore, we found that Cys⁶² is critical for AdpA sensing sulfane sulfur.
 369 Sulfane sulfur treatment leads to a sulphydration modification in Cys⁶² (Cys⁶²-SSH).
 370 This modification is not observed in the other three cysteine residues of AdpA. The
 371 AlphaFold 2 predicted structure shows that the thiol of Cys⁶² is accessible to solution
 372 while the other thiols are not, which may explains why Cys⁶² is easily sulphydrated by
 373 sulfane sulfur. However, it's still unknown how this post-translational modification
 374 affects AdpA configuration. Since Cys⁶² locates in the dimerization domain, it may
 375 affect the dimerization and then alter the AdpA binding to its cognate DNA.

376 In recent years, a few transcription factors that can be modified by sulfane sulfur
 377 have been identified from different microorganisms. These transcription factors can
 378 be categorized into two groups. Group I consists of specific regulators for genes
 379 related to sulfur metabolism, including BigR (36), CstR (37), FisR (38), CsoR (23),
 380 and SqrR (39, 40). They control the expression of sulfane sulfur oxidation enzyme
 381 PDO and sulfane sulfur transferase RhoD (BigR is in this case). Since H₂S oxidation
 382 enzyme SQR often locates in the same operon with PDO and RhoD, they also
 383 controls SQR expression (CstR, FisR, and SqrR are in this case) (41). Group I
 384 regulators can sense the intracellular level of sulfane sulfur via their cysteine residues:
 385 when sulfane sulfur level is high, cysteine residues are sulphydrated to form RS_nH
 386 ($n \geq 2$) or RS_nR ($n \geq 3$) bond, which leads configuration change of the regulator and
 387 subsequently high expression of PDO and other genes, and then sulfane sulfur level is
 388 decreased through oxidizing to sulfite (19). Therefore, Group I regulators mainly

389 function as managers to maintain the homeostasis of intracellular sulfane sulfur.

390 Group II includes global or multi-functional transcription factors currently
391 including MgrA (21), MexR (42), and OxyR (43). MgrA is a global virulence
392 regulator of *Staphylococcus aureus*. It sense intracellular level of sulfane sulfur to
393 regulate the expression of virulence factors (21). MexR controls the multiple
394 antibiotic resistance process in *Pseudomonas aeruginosa*, it senses intracellular
395 sulfane sulfur to regulate the expression of *mexAB-oprM* multidrug efflux operon (42).
396 OxyR is a global anti-oxidation regulator in many bacteria. Recently it was found that
397 OxyR also senses sulfane sulfur and controls expression of sulfane sulfur reducing
398 enzymes (43). Same as Group I, Group II regulators also sense sulfane sulfur via their
399 cysteine residues.

400 AdpA is deemed as a Group II regulator since it senses sulfane sulfur and
401 accordingly adjust the ACT production and spore formation in *S. coelicolor*.
402 Bioinformatics analyses indicate that AdpA and its Cys residues are highly conserved
403 in *Streptomyces* spp., and further investigations of this protein and its homologues
404 should provide insights into how sulfane sulfur regulates the production of secondary
405 metabolites and morphological developments in this genus. The widespread existence
406 of AdpA implies that sulfane sulfur may play a wide range of regulatory functions in
407 *Streptomyces*, providing unlimited possibilities for sulfane sulfur as a signal molecule
408 to stimulate increased production of important secondary metabolites, such as
409 antibiotics, anti-tumor drugs, immunosuppressants, and antibiotics.

410

411 **MATERIALS AND METHODS**

412 **Bacterial strains, plasmids and growth conditions.** All strains and plasmids used or
413 constructed in this work are summarized in Table S1.

414 *Streptomyces* strains were cultivated at 30°C on MS (mannitol soya flour) solid
415 medium (44), YBP (yeast-beef-peptone) solid or liquid medium (45) were used for
416 different experiments including spore suspension preparation, intergeneric
417 conjugation, growth assay, RNA isolation and phenotypic observation. All *E. coli*
418 strains were cultured at 37°C on solid or liquid Luria-Bertani (LB) medium. *E. coli*
419 DH5 α and *E. coli* BL21 (DE3) strains were used as hosts for plasmid construction and
420 protein expression, respectively. *E. coli* ET12567 (pUZ8002) was used as a media for
421 transferring nonmethylated DNA to *Streptomyces*. When required, ampicillin (100
422 μ g/mL), apramycin (50 μ g/mL), chloramphenicol (25 μ g/mL), kanamycin (50 μ g/mL),
423 hygromycin (50 μ g/mL), or nalidixic acid (25 μ g/mL) were added into the medium.

424

425 **Preparation of sulfane sulfur species and other sulfur containing compounds.**

426 Sodium hydrosulfide (NaHS, H₂S donor) and thiosulfate were purchased from Sigma
427 Aldrich. S₈ solution was freshly prepared by dissolving excess sulfur powder in
428 acetone to reach saturation. The stock solution of HS_nH (hydrogen polysulfides) was
429 prepared by mixing sulfur powder, NaOH, and NaHS (40 mM of each chemical) in
430 degassed distilled water at 30°C till the powder was completely dissolved as previously
431 described (42, 46). The concentrations of HS_nH was determined with a cyanolysis
432 method (47) and calibrated by using thiosulfate as the standard. The entire process

433 was performed under anaerobic condition.

434

435 **Construction of *S. coelicolor* $\Delta adpA$.** All primers used in this experiment are listed in
 436 supplemental Table S2. The strain $\Delta adpA$ was constructed and generated using a
 437 homologous recombination method (48). Briefly, a 939 bp region was deleted from
 438 ORF of *adpA*, leaving the upstream 168 bp (relative to the start codon) and the
 439 downstream 90 bp (relative to the stop codon) coding sequence of *adpA*. The
 440 knockout region was replaced by the *apramycin* resistance gene. The conjugation
 441 transfer was accomplished using the methylation-sensitive strain *E. coli*
 442 ET12567/pUZ8002 (containing the mutant plasmid pJTU-*adpA*) and *S. coelicolor*
 443 M145 following a previously reported protocol (49). The deletion mutant was verified
 444 by resistance screening and colony PCR with primers VeradpA-F/R.

445

446 **Construction of $\Delta adpA::adpA$, $\Delta adpA::pMS82$, $\Delta adpA::adpA_{C62S}$,**
 447 **$\Delta adpA::adpA_{C126S}$, $\Delta adpA::adpA_{C187S}$ and $\Delta adpA::adpA_{C307S}$.** A DNA fragment
 448 carrying the *adpA* ORF (1197 bp) and its promoter (500 bp) was obtained using PCR
 449 amplification and connected to the Φ BT1 integrative vector pMS82 (50) to generate
 450 *adpA*-complemented plasmid pMS82-*adpA* (Table S1), which was then integrated into
 451 the *attP* site of $\Delta adpA$ genome by intergeneric conjugation. To construct negative
 452 control strains, empty pMS82 vector was also transformed into $\Delta adpA$, these
 453 derivative strains were selected and confirmed by PCR and DNA sequencing.

454 In order to gain complementary strains with cysteine mutations, we adopted a point

455 mutation strategy (51) based on the complementing plasmid pMS82-*adpA*, and
 456 obtained the resulting plasmids pMS82-*adpA*_{C62S}, pMS82-*adpA*_{C126S},
 457 pMS82-*adpA*_{C187S} and pMS82-*adpA*_{C307S}. The same method was used to obtain
 458 complementary strains $\Delta adpA::adpA_{C62S}$, $\Delta adpA::adpA_{C126S}$, $\Delta adpA::adpA_{C187S}$ and
 459 $\Delta adpA::adpA_{C307S}$. The primers used in this process are shown in Table S2.
 460
 461 **AdpA protein overexpression, purification and mutation.** To construct the AdpA
 462 expression strain, the coding sequence of *adpA* was amplified from wt genomic DNA
 463 with primers ExadpA-F/R. The PCR product was purified and ligated into the pET15b
 464 vector with a C-terminal His tag to create plasmid pET-AdpA by using the
 465 ClonExpressTM II One Step Cloning Kit (TaKaRa). The plasmid was transformed into
 466 *E. coli* BL21 (DE3) cells, which were grown in LB medium at 37°C to an OD₆₀₀ of
 467 about 0.6, and then a total of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)
 468 was added and an additional overnight cultivation was continued at 16°C. Cultures
 469 were collected by centrifugation and disrupted through a pressure cell homogenizer
 470 (SPCH-18) in sonication buffer (50 mM NaH₂PO₄, 250 mM NaCl, 20 mM imidazole,
 471 pH 8.0), 1 mM DTT was added before breaking the cells. Purification of the AdpA
 472 His-tagged proteins were performed with Ni-NTA-Sefinose column (Sangon) as
 473 described previously (23). The eluted fractions containing this protein were
 474 exchanged to a buffer (50 mM NaH₂PO₄, 50 mM NaCl, 10% of glycerol, pH 8.0) via
 475 PD-10 desalting column (GE), protein purification process was operated in an
 476 anaerobic glove box, the anaerobic box first exchanges gas with N₂, and then the

anaerobic box is filled with mixed gas (N₂, 85%; H₂, 10%; CO₂, 5%) to keep the inside free of oxygen examined by a gas detector (ADKS-4, EDKORS, China). The purity of the protein was assessed by SDS-PAGE gel and its concentration was determined using the BCA Protein Assay Reagent (Thermo Fisher Scientific). AdpA cysteine mutant proteins (C62S, C126S, C187S and C307S) plasmids were constructed using the modified QuikChange™ method (51). We used the same method to express, purify and analyze these variants.

Electrophoretic mobility shift assay (EMSA). The DNA probes containing AdpA binding sequences were amplified from its target promoter region on the genomic DNA. Different sulfane sulfur compounds were reacted with the same amount of AdpA protein or cysteine mutant proteins in the binding buffer (20 mM Tris-HCl, 2 mM EDTA, 20 mM KCl, 0.5 mM DTT, pH 8.0) at room temperature for 20 minutes, and then the same amount of DNA was added to react at 30°C for 20 minutes, the samples were separated on an 8% nondenaturing polyacrylamide gel at 120V for 2 hour in ice (52), the gel was dyed with SYBR Green I (Sangon) for 20 minutes (38), All images were captured with a FlourChemQ system (Alpha Innotech).

RNA preparation, RT-PCR and RT-qPCR. To extract RNA, spores (2×10^7) of wt and $\Delta adpA$ strains were inoculated into the liquid YBP medium and incubated at 30°C with shaking (220 rpm) for 36 h to mid-exponential phase. HS_nH (400 μM) or S₈ (400 μM) was added. After 30 minutes of continued cultivation, these mycelia were

499 harvested by centrifugation for 10 min, grounded into powder with liquid nitrogen.
500 Similarity, the cultures of wt, $\Delta adpA$ and Δpdo were collected on YBP plates at
501 indicated times. All RNA were isolated with *SteadyPure* Universal RNA Extraction
502 Kit (Accurate Biology) following manufacturer's instructions, and their quality and
503 concentration were determined by a NanoDrop ND-1000 (Thermo Fisher). Turbo
504 DNaseI (Invitrogen) was utilized to remove genomic DNA. RT-PCR was carried out
505 using a reverse transcriptase kit (Invitrogen) following manufacturer's
506 recommendations. RT-qPCR was performed using SYBR Premix Ex Taq (Takara).
507 Roche LightCycler 480 thermal cycler was used to assess the melting curve and
508 quantification of the PCR products, and detailed thermal cycling steps was carried out
509 as the protocol previous described (53). The expression of *hrdB* mRNA was used as
510 the internal standard to normalize the relative quantities of cDNA. The relative
511 expression abundance of the target gene were analyzed using a relative quantification
512 method of $2^{-\Delta Ct}$ (test gene-*hrdB*), three independent replicates were performed in
513 parallel.

514

515 **Phenotypic analysis and actinorhodin production assay.** *S. coelicolor* strains were
516 cultured on solid YBP medium at 30°C for phenotypic observation and imaged at
517 indicated times. ACT production measurement was performed as described previously
518 (23, 54, 55). Briefly, *Streptomyces* strains were incubated on YBP medium for 7 or 10
519 days, mycelia were harvested from the plate. KOH (1 M final concentration) was
520 added to treat the mycelia for 4 h. Then the mixtures were centrifuged to collect the

521 supernatant, in which ACT concentration was determined by a spectrophotometer.

522 Three independent biological experiments were replicated.

523

524 **Construction and testing of EGFP reporter systems.** To construct the reporter

525 plasmids, promoter fragments (nucleotides -400 to -1 upstream from gene

526 translational start codon of *actII-4* and nucleotides -460 to -1 upstream from gene

527 translational start codon of *wblA*) were amplified by PCR using primers

528 pMS82-*actII-4p-egfp* S1-F/R and pMS82-*wblAp-egfp* S1-F/R (Table S2), respectively.

529 Then these promoter fragments and a DNA fragment encompassing the *egfp* gene

530 (coding for a green fluorescent protein from pEGFP-C2) were mixed and cloned into

531 the pMS82 vector, yielding pMS82-*actII-4p-egfp* and pMS82-*wblAp-egfp*,

532 respectively. Next, we introduced these reporter plasmids into wt and $\Delta adpA$ via

533 conjugation with non-methylating *E. coli* ET12567 (pUZ8002), respectively.

534 Exconjugants with hygromycin-resistant strains were screened and checked by PCR

535 using specific primers (Table S2).

536 Strains containing the reporter plasmid were pre-cultured in liquid YBP medium for

537 36 h at 30°C. Subsequently, equal amount mycelia of each strain was transferred to

538 the fluted bottle, and inducers (HS_nH and S₈) were added. After 60 min of induction,

539 the bacteria were collected by centrifugation, and mycelia were resuspended in 200 μ l

540 PBS buffer (OD₄₅₀=2). EGFP fluorescence was measured using the microplate reader

541 Synergy H1. The excitation wavelength and emission wavelength were set to 485 nm

542 and 515 nm, respectively. The EGFP fluorescence intensity was normalized against

543 cell density (fluorescence/OD₄₅₀ of mycelia).

544

545 **LC-MS/MS analysis of AdpA.** The analysis was performed following a previous
 546 report (23). Freshly purified protein AdpA was treated with 10 fold amounts of HS_nH
 547 or DTT. After reacting at room temperature for 40 minutes. The reacted protein was
 548 treated with denaturing buffer (0.5 M Tris-HCl, 2.75 mM EDTA, 6 M guanadine-HCl,
 549 pH 8.0) containing 1 M iodoacetamide (IAM). The treatment was carried out in dark
 550 for 1 h, then sample was digested with trypsin (1:25, w/w) at 37°C for 20 h. The
 551 digestion products were filtered by C18 Zip-Tip (Millipore) and vacuum dried.
 552 Obtained peptides were resuspended in 10 µl ddH₂O.

553 The Prominence nano-LC system (Shimadzu) equipped with a custom-made silica
 554 column (75 µm × 15 cm) packed with 3-µm Reprosil-Pur 120 C18-AQ was used.
 555 Positive electrospray ionization was performed and the ions were scanned via
 556 LTQ-Orbitrap Velos Pro CID mass spectrometer (Thermo Scientific); the data were
 557 analyzed by a data-dependent acquisition mode with Xcalibur 2.2.0 software (Thermo
 558 Scientific). Full-scan MS spectra (from 400 to 1800 m/z) was detected and assessed
 559 by the Orbitrap at a resolution of 60,000 at 400 m/z.

560

561 **AdpA structure modeling.** The AlphaFold 2 algorithm (56) was used to predict the
 562 tertiary structure of AdpA. This method used the custom multiple sequence alignment
 563 (MSA) option and accessed by the Colab server on GitHub
 564 (<https://github.com/sokrypton/ColabFold>). Structural model of AdpA was analyzed

565 and visualized with PyMOL.

566

567 **Fluorescence polarization (FP) analysis.** FP analysis experiments were performed

568 following a reported protocol (57). DNA probes were amplified by PCR and labelled

569 by 5'-FAM (Carboxyfluorescein) (Sangon). Purified AdpA (treated with 0.5 mM

570 HS_nH for 10 min or not) was diluted to different concentrations (0.01 μM~22.5 μM).

571 The reaction buffer contained 10 mM Tris-HCl and 75 mM NaCl, pH 7.5. After

572 mixing diluted AdpA and labelled DNA in the reaction buffer, the solution was

573 incubated at 37°C for 15 min in the dark. The fluorescence was detected with BioTek

574 Synergy HT. The K_D value was calculated using GraphPad Prism 5 software.

575

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579

580 **DECLARATION OF COMPETING INTEREST**

581 The authors declare no conflict of interest regarding the publication of this article.

582

583 **REFERENCE**

584 1. Sanchez J, Yague P, Manteca A. 2012. New Insights in *Streptomyces*

585 Fermentations. Ferment Technol 1:e105.

586 2. Olmos E, Mehmood N, Husein LH, Goergen JL, Fick M, Delaunay S. 2013.

587 Effects of bioreactor hydrodynamics on the physiology of *Streptomyces*.
588 Bioproc Biosyst Eng 36:259-272.

589 3. Hasani A, Kariminik A, Issazadeh K. 2014. *Streptomyces*: characteristics and
590 their antimicrobial activities. Int J Adv Biol Biomed Res 2:63-75.

591 4. Lee N, Hwang S, Kim J, Cho S, Palsson B, Cho BK. 2020. Mini review:
592 Genome mining approaches for the identification of secondary metabolite
593 biosynthetic gene clusters in *Streptomyces*. Comput Struct Biotech
594 18:1548-1556.

595 5. Honma S, Ito S, Yajima S, Sasaki Y. 2021. Nitric oxide signaling for
596 actinorhodin production in *Streptomyces coelicolor* A3(2) via the DevS/R
597 two-component system. Appl Environ Microb 87:e00480-21.

598 6. Lee N, Hwang S, Kim W, Lee Y, Kim JH, Cho S, Kim HU, Yoon YJ, Oh MK,
599 Palsson BO, Cho BK. 2021. Systems and synthetic biology to elucidate
600 secondary metabolite biosynthetic gene clusters encoded in *Streptomyces*
601 genomes. Nat Prod Rep 38:1330-1361.

602 7. Ohnishi Y, Yamazaki H, Kato JY, Tomono A, Horinouchi S. 2005. AdpA, a
603 central transcriptional regulator in the A-factor regulatory cascade that leads to
604 morphological development and secondary metabolism in *Streptomyces*
605 *griseus*. Biosci Biotech Bioch 69:431-439.

606 8. den Hengst CD, Tran NT, Bibb MJ, Chandra G, Leskiw BK, Buttner MJ. 2010.
607 Genes essential for morphological development and antibiotic production in
608 *Streptomyces coelicolor* are targets of BldD during vegetative growth. Mol

- 609 Microbiol 78:361-379.
- 610 9. Yan H, Lu XR, Sun D, Zhuang S, Chen Q, Chen Z, Li JL, Wen Y. 2020. BldD,
611 a master developmental repressor, activates antibiotic production in two
612 *Streptomyces* species. Mol Microbiol 113:123-142.
- 613 10. Setinova D, Smidova K, Pohl P, Music I, Bobek J. 2018. RNase
614 III-binding-mRNAs revealed novel complementary transcripts in *Streptomyces*.
615 Front Microbiol 8:2693.
- 616 11. Takano E, Tao M, Long F, Bibb MJ, Wang L, Li W, Buttner MJ, Bibb MJ,
617 Deng ZX, Chater KF. 2003. A rare leucine codon in *adpA* is implicated in the
618 morphological defect of *bldA* mutants of *Streptomyces coelicolor*. Mol
619 Microbiol 50:475-486.
- 620 12. Kato J, Ohnishi Y, Horinouchi S. 2005. Autorepression of AdpA of the
621 AraC/XylS family, a key transcriptional activator in the A-factor regulatory
622 cascade in *Streptomyces griseus*. J Mol Biol 350:12-26.
- 623 13. Rabyk M, Yushchuk O, Rokytskyy I, Anisimova M, Ostash B. 2018. Genomic
624 insights into evolution of AdpA family master regulators of morphological
625 differentiation and secondary metabolism in *Streptomyces*. J Mol Evol
626 86:204-215.
- 627 14. Lau N, Pluth MD. 2019. Reactive sulfur species (RSS): persulfides,
628 polysulfides, potential, and problems. Curr Opin Chem Biol 49:1-8.
- 629 15. Filipovic MR, Zivanovic J, Alvarez B, Banerjee R. 2018. Chemical biology of
630 H₂S signaling through persulfidation. Chem Rev 118:377-461.

- 631 16. Fukuto JM, Ignarro LJ, Nagy P, Wink DA, Kevil CG, Feelisch M,
632 Cortese-Krott MM, Bianco CL, Kumagai Y, Hobbs AJ, Lin J, Ida T, Akaike T.
633 2018. Biological hydropersulfides and related polysulfides - a new concept
634 and perspective in redox biology. FEBS Lett 592:2140-2152.
- 635 17. Toohey JJ, Cooper AJL. 2014. Thiosulfoxide (sulfane) sulfur: new chemistry
636 and new regulatory roles in biology. Molecules 19:12789-12813.
- 637 18. Wang SSS, Chen YHH, Chen N, Wang LJJ, Chen DXX, Weng HLL, Dooley S,
638 Ding HGG. 2017. Hydrogen sulfide promotes autophagy of hepatocellular
639 carcinoma cells through the PI3K/Akt/mTOR signaling pathway. Cell Death
640 Dis 8:e2688.
- 641 19. Xin YF, Gao R, Cui FF, Lü CJ, Liu HL, Liu HW, Xia YZ, Xun LY. 2020. The
642 heterotrophic bacterium *Cupriavidus pinatubonensis* JMP134 oxidizes sulfide
643 to sulfate with thiosulfate as a key intermediate. Appl Environ Microb
644 86:e01835-20.
- 645 20. Wunder LC, Aromokeye DA, Yin XR, Richter-Heitmann T, Willis-Poratti G,
646 Schnakenberg A, Otersen C, Dohrmann I, Romer M, Bohrmann G, Kasten S,
647 Friedrich MW. 2021. Iron and sulfate reduction structure microbial
648 communities in (sub-)Antarctic sediments. Isme J.
- 649 21. Peng H, Zhang YX, Palmer LD, Kehl-Fie TE, Skaar EP, Trinidad JC, Giedroc
650 DP. 2017. Hydrogen sulfide and reactive sulfur species impact proteome
651 S-sulphydration and global virulence regulation in *Staphylococcus aureus*. Acs
652 Infect Dis 3:744-755.

- 653 22. Liu DX, Zhang JJ, Lü CJ, Xia YZ, Liu HW, Jiao NZ, Xun LY, Liu JH. 2020.
654 *Synechococcus* sp. strain PCC7002 uses sulfide:quinone oxidoreductase to
655 detoxify exogenous sulfide and to convert endogenous sulfide to cellular
656 sulfane sulfur. *Mbio* 11:e03420-19.
- 657 23. Lu T, Cao Q, Pang XH, Xia YZ, Xun LY, Liu HW. 2020. Sulfane
658 sulfur-activated actinorhodin production and sporulation is maintained by a
659 natural gene circuit in *Streptomyces coelicolor*. *Microb Biotechnol*
660 13:1917-1932.
- 661 24. Park SS, Yang YH, Song E, Kim EJ, Kim WS, Sohng JK, Lee HC, Liou KK,
662 Kim BG. 2009. Mass spectrometric screening of transcriptional regulators
663 involved in antibiotic biosynthesis in *Streptomyces coelicolor* A3(2). *J Ind*
664 *Microbiol Biot* 36:1073-1083.
- 665 25. Liu G, Chater KF, Chandra G, Niu GQ, Tan HR. 2013. Molecular regulation
666 of antibiotic biosynthesis in *Streptomyces*. *Microbiol Mol Biol R* 77:112-143.
- 667 26. Arias P, Fernandez-Moreno MA, Malpartida F. 1999. Characterization of the
668 pathway-specific positive transcriptional regulator for actinorhodin
669 biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. *J*
670 *Bacteriol* 181:6958-6968.
- 671 27. Lee HN, Kim JS, Kim P, Lee HS, Kim ES. 2013. Repression of antibiotic
672 downregulator WblA by AdpA in *Streptomyces coelicolor*. *Appl Environ*
673 *Microb* 79:4159-4163.
- 674 28. Wolanski M, Donczew R, Kois-Ostrowska A, Masiewicz P, Jakimowicz D,

- 675 Zakrzewska-Czerwinska J. 2011. The level of AdpA directly affects
676 expression of developmental genes in *Streptomyces coelicolor*. J Bacteriol
677 193:6358-6365.
- 678 29. Mustafa AK, Gadalla MM, Sen N, Kim S, Mu WT, Gazi SK, Barrow RK,
679 Yang GD, Wang R, Snyder SH. 2009. H₂S signals through protein
680 S-sulfhydration. Sci Signal 2:ra72.
- 681 30. Paul BD, Snyder SH. 2015. Protein sulfhydration. Method Enzymol
682 555:79-90.
- 683 31. Bu XL, Weng JY, He BB, Xu MJ, Xu J. 2019. A novel AdpA homologue
684 negatively regulates morphological differentiation in *Streptomyces*
685 *xiamenensis* 318. Appl Environ Microb 85:e03107-18.
- 686 32. Kang YJ, Wang YY, Hou BB, Wang RD, Ye J, Zhu XY, Wu HZ, Zhang HZ.
687 2019. AdpA_{lin}, a pleiotropic transcriptional regulator, is involved in the
688 cascade regulation of lincomycin biosynthesis in *Streptomyces lincolnensis*.
689 Front Microbiol 10:2428.
- 690 33. Yushchuk O, Ostash I, Vlasiuk I, Gren T, Luzhetskyy A, Kalinowski J,
691 Fedorenko V, Ostash B. 2018. Heterologous AdpA transcription factors
692 enhance landomycin production in *Streptomyces cyanogenus* S136 under a
693 broad range of growth conditions. Appl Microbiol Biot 102:8419-8428.
- 694 34. Xu JJ, Zhang JH, Zhuo JM, Li Y, Tian YQ, Tan HR. 2017. Activation and
695 mechanism of a cryptic oviedomycin gene cluster via the disruption of a
696 global regulatory gene, adpA, in *Streptomyces ansochromogenes*. J Biol Chem

697 292:19708-19720.

698 35. Mao XM, Luo S, Zhou RC, Wang F, Yu P, Sun N, Chen XX, Tang Y, Li YQ.
699 2015. Transcriptional regulation of the daptomycin gene cluster in
700 *Streptomyces roseosporus* by an autoregulator, AtrA. J Biol Chem
701 290:7992-8001.

702 36. de Lira NPV, Pauletti BA, Marques AC, Perez CA, Caserta R, de Souza AA,
703 Vercesi AE, Leme AFP, Benedetti CE. 2018. BigR is a sulfide sensor that
704 regulates a sulfur transferase/dioxygenase required for aerobic respiration of
705 plant bacteria under sulfide stress. Sci Rep-Uk 8:3508.

706 37. Luebke JL, Shen JC, Bruce KE, Kehl-Fie TE, Peng H, Skaar EP, Giedroc DP.
707 2014. The CsoR-like sulfurtransferase repressor (CstR) is a persulfide sensor
708 in *Staphylococcus aureus*. Mol Microbiol 94:1343-1360.

709 38. Li HJ, Li J, Lü CJ, Xia YZ, Xin YF, Liu HL, Xun LY, Liu HW. 2017. FisR
710 activates σ^{54} -dependent transcription of sulfide-oxidizing genes in
711 *Cupriavidus pinatubonensis* JMP134. Mol Microbiol 105:373-384.

712 39. Shimizu T, Shen JC, Fang MX, Zhang YX, Hori K, Trinidad JC, Bauer CE,
713 Giedroc DP, Masuda S. 2017. Sulfide-responsive transcriptional repressor
714 SqrR functions as a master regulator of sulfide-dependent photosynthesis. P
715 Natl Acad Sci USA 114:2355-2360.

716 40. Capdevila DA, Walsh BJC, Zhang YF, Dietrich C, Gonzalez-Gutierrez G,
717 Giedroc DP. 2021. Structural basis for persulfide-sensing specificity in a
718 transcriptional regulator. Nat Chem Biol 17:65-70.

- 719 41. Giedroc DP. 2017. A new player in bacterial sulfide-inducible transcriptional
720 regulation. *Mol Microbiol* 105:347-352.
- 721 42. Xuan GH, Lü CJ, Xu HW, Chen ZG, Li K, Liu HL, Liu HW, Xia YZ, Xun LY.
722 2020. Sulfane Sulfur is an intrinsic signal activating MexR-regulated antibiotic
723 resistance in *Pseudomonas aeruginosa*. *Mol Microbiol* 114:1038-1048.
- 724 43. Hou NK, Yan ZZ, Fan KL, Li HJ, Zhao R, Xia YZ, Xun LY, Liu HW. 2019.
725 OxyR senses sulfane sulfur and activates the genes for its removal in
726 *Escherichia coli*. *Redox Biol* 26:101293.
- 727 44. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (ed). 2000. *Practical*
728 *Streptomyces* Genetics. Norwich: John Innes Foundation.,
- 729 45. Zhu YP, Lu T, Zhang J, Zhang PP, Tao MF, Pang XH. 2020. A novel XRE
730 family regulator that controls antibiotic production and development in
731 *Streptomyces coelicolor*. *Appl Microbiol Biot* 104:10075-10089.
- 732 46. Xin YF, Liu HL, Cui FF, Liu HW, Xun LY. 2016. Recombinant iwith sulfide:
733 quinone oxidoreductase and persulfide dioxygenase rapidly oxidises sulfide to
734 sulfite and thiosulfate via a new pathway. *Environ Microbiol* 18:5123-5136.
- 735 47. Flavin M. 1962. Microbial transsulfuration: the mechanism of an enzymatic
736 disulfide elimination reaction. *J Biol Chem* 237:768-777.
- 737 48. Lu T, Zhu YP, Zhang PP, Sheng DH, Cao GX, Pang XH. 2018. SCO5351 is a
738 pleiotropic factor that impacts secondary metabolism and morphological
739 development in *Streptomyces coelicolor*. *Fems Microbiol Lett* 365:fny150.
- 740 49. Zhu YP, Xu WH, Zhang J, Zhang PP, Zhao ZL, Sheng DH, Ma W, Zhang YZ,

741 Bai LQ, Pang XH. 2020. A hierarchical network of four regulatory genes
742 controlling production of the polyene antibiotic candicidin in *Streptomyces* sp.
743 Strain FR-008. Appl Environ Microb 86:e00055-20.

744 50. Gregory MA, Till R, Smith MCM. 2003. Integration site for *Streptomyces*
745 phage phiBT1 and development of site-specific integrating vectors. J Bacteriol
746 185:5320-5323.

747 51. Xia YZ, Chu WQ, Qi QS, Xun LY. 2015. New insights into the QuikChangeTM
748 process guide the use of Phusion DNA polymerase for site-directed
749 mutagenesis. Nucleic Acids Res 43:e12.

750 52. Zhu YP, Zhang PP, Zhang J, Xu WH, Wang XY, Wu LL, Sheng DH, Ma W,
751 Cao GX, Chen XA, Lu YH, Zhang YZ, Pang XH. 2019. The developmental
752 regulator MtrA binds GlnR boxes and represses nitrogen metabolism genes in
753 *Streptomyces coelicolor*. Mol Microbiol 112:29-46.

754 53. Zhu YP, Zhang PP, Lu T, Wang XY, Li AY, Lu YH, Tao MF, Pang XH. 2021.
755 Impact of MtrA on phosphate metabolism genes and the response to altered
756 phosphate conditions in *Streptomyces*. Environ Microbiol 23:6907-6923.

757 54. Liu M, Zhang PP, Zhu YP, Lu T, Wang YM, Cao GX, Shi M, Chen XL, Tao
758 MF, Pang XH. 2019. Novel two-component system MacRS is a pleiotropic
759 regulator that controls multiple morphogenic membrane protein genes in
760 *Streptomyces coelicolor*. Appl Environ Microb 85:e02178-18.

761 55. Zhu YP, Zhang PP, Zhang J, Wang J, Lu YH, Pang XH. 2020. Impact on
762 multiple antibiotic pathways reveals MtrA as a master regulator of antibiotic

763 production in *Streptomyces* spp. and potentially in other actinobacteria. Appl
764 Environ Microb 86:e01201-20.

765 56. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O,
766 Tunyasuvunakool K, Bates R, Zidek A, Potapenko A, Bridgland A, Meyer C,
767 Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler
768 J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M,
769 Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW,
770 Kavukcuoglu K, Kohli P, Hassabis D. 2021. Highly accurate protein structure
771 prediction with AlphaFold. Nature 596:583-589.

772 57. Zhang FY, Li BQ, Dong HJ, Chen M, Yao S, Li JW, Zhang HH, Liu XG,
773 Wang HW, Song NN, Zhang KD, Du N, Xu SJ, Gu LC. 2020. YdiV regulates
774 *Escherichia coli* ferric uptake by manipulating the DNA-binding ability of Fur
775 in a SlyD-dependent manner. Nucleic Acids Res 48:9571-9588.

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