

RESEARCH ARTICLE

# In conditions of over-expression, Wbll, a WhiB-like transcriptional regulator, has a positive impact on the weak antibiotic production of *Streptomyces lividans* TK24

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## Abstract

Regulators of the WhiB-like (*wbl*) family are playing important role in the complex regulation of metabolic and morphological differentiation in *Streptomyces*. In this study, we investigated the role of *wbll*, a member of this family, in the regulation of secondary metabolite production in *Streptomyces lividans*. The over-expression of *wbll* was correlated with an enhanced biosynthesis of undecylprodigiosin and actinorhodin and with a reduction of the biosynthesis of yCPK and of the grey spore pigment encoded by the *whiE* locus. Five regulatory targets of Wbll were identified using *in vitro* formaldehyde crosslinking and confirmed by EMSA and qRT-PCR. These included the promoter regions of *wbll* itself, two genes of the ACT cluster (*actVA3* and the intergenic region between the divergently orientated genes *actII-1* and *actII-2*) and that of *wblA*, another member of the Wbl family. Quantitative RT-PCR analysis indicated that the expression of *actVA3* encoding a protein of unknown function as well as that of *actII-1*, a TetR regulator repressing the expression of *actII-2*, encoding the ACT transporter, were down regulated in the Wbll over-expressing strain. Consistently the expression of the transporter *actII-2* was up-regulated. The expression of WblA, that is known to have a negative impact on ACT biosynthesis, was strongly down regulated in the Wbll over-expressing strain. These data are consistent with the positive impact that Wbll over-expression has on ACT biosynthesis. The latter might result from direct activation of ACT biosynthesis and export and from repression of the expression of WblA, a likely indirect, repressor of ACT biosynthesis.

analysis, decision to publish, or preparation of the manuscript.

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## Introduction

*Streptomyces* are Gram-positive, filamentous soil bacteria of considerable biotechnological importance. Indeed this genus produces two thirds of all known antibiotics as well as other bio-active molecules, including antitumor agents, immune-suppressants, apoptosis inducers and antifungals, herbicides, insecticides etc. . . used in medicine or agriculture [1]. These bacteria are characterized by a complex developmental cycle that starts, when the nutritional conditions are favorable, by the germination of spores that develop into a substrate mycelium. Subsequently, some still poorly defined signals of nutrient limitation, are thought to trigger the development of aerial hyphae from the substrate mycelium. The tip ends of the aerial hyphae differentiate into uni-genomic spores and the production of a grey pigment encoded by the *whiE* locus accompanied the complete differentiation process [2,3]. This complex morphological development is mainly under the control of the *bld* and *whi* genes, that are required for the formation of aerial mycelium and spores, respectively [2,3].

The complex *bld* signaling cascade has been extensively studied leading to the characterization of the BldA, BldD, BldH and BldN regulons [4–8]. A signal molecule c-di-GMP was recently shown to induce the dimerization of the regulator BldD that is necessary to activate its DNA-binding activity [9,10]. The binding of BldD results in the repression of the sporulation genes during vegetative growth leading to a delay in the differentiation process [9,10]. One of the genes under the negative control of BldD is the  $\sigma$  factor, *whiG* [4,11,12]. The latter is necessary for the expression of numerous genes of the *whi* cascade, including that of the regulators *whiA*, *whiH*, *whiI* but not *whiB* [12–15]. Whi regulators are named from the white color of aerial hyphae following the mutation/disruption of their cognate encoding genes. They are known to play key roles in the differentiation of aerial hyphae into mature spores [2]. WhiB, regarded as the founding member of the WhiB-like (Wbl) family proteins, is one of the Whi regulators that is necessary for the septation steps preceding sporulation [16,17]. A recent genome-wide study demonstrated that WhiB is a transcriptional factor that binds, in cooperation with WhiA, upstream of nearly 240 transcription units required for developmental cell division [18]. Wbl proteins usually contain an unconventional helix-turn-helix motif and a [4Fe-4S] iron-sulfur cluster that have the ability to detect redox changes and regulate gene expression accordingly [19]. Such redox sensing clusters have been shown to play diverse and critical roles in actinobacterial biology, including morphological differentiation, antibiotic production, antibiotic resistance and pathogenesis [16,20–22]. There are 14 WhiB-like (Wbl) regulators present in *S. coelicolor*, 11 Wbl proteins are encoded by chromosomal genes, and 3 are encoded by the plasmid SCP1 [23]. *wblA* is the most extensively studied of these regulators. It was shown to play important role in the early stage of aerial hyphal development [23,24] and to have a negative impact on oxidative stress response [25,26] and antibiotic production [24]. In contrast WhiD was shown to be essential for pre-spore maturation [27–30].

In this study we characterized another member of the Wbl gene family, WbII (SCO5046). This gene was previously shown to be a target of SCO3201, a regulator of the TetR family, whose overexpression led to strong repression of both antibiotic production and sporulation in *S. coelicolor* [31]. We demonstrated that the over-expression of the homologue of WbII in *S. lividans* TK24, greatly enhanced the weak ability of this strain to synthesize undecylprodigiosin (RED) and actinorhodin (ACT), peptidyl and polyketide secondary metabolites, respectively whereas it had a negative impact on the biosynthesis of yCPK, a type I polyketide as well as of the grey spore polyketide pigment encoded by the *whiE* locus. Regulatory targets of WbII were identified by formaldehyde cross linking and confirmed by EMSA and qRT-PCR. WbII constitutes a new player in the complex regulatory network governing secondary metabolite production and morphological differentiation in *Streptomyces*. A regulatory model consistent with

the impact that WbII over-expression/deletion has on the expression of its targets and on antibiotic production is proposed and discussed. This model clarifies the hierarchical relationships between WbII and WbIA.

## Materials and methods

### Bacterial strains, media and culture conditions

*S. lividans* TK24 (*str-6*, SLP2<sup>-</sup>, SLP3<sup>-</sup>) was used in this study. *Escherichia coli* DH5 $\alpha$  and BL21 were used as the hosts for routine subcloning and protein expression, respectively. SFM [32] and GYM [33] media were used for spore collection and the assessment of spore grey pigment production, respectively. R2YE medium, traditionally used for protoplast regeneration, was used for solid-grown cultures of *S. lividans*. To assess antibiotic production, 10<sup>7</sup> spores of various *Streptomyces* strains were spread on top of cellophane discs on R2YE agar medium. When necessary, ampicillin, kanamycin or apramycin were added to the culture medium at 50  $\mu$ g/ml, whereas thiostrepton was added to R2YE liquid medium at 5  $\mu$ g/ml. Unless otherwise stated, *E. coli* and *Streptomyces* strains were incubated at 37°C and 28°C, respectively, and a shaking speed of 220 rpm was maintained for liquid culture.

### Construction of *S. lividans* TK24 strains overexpressing or disrupted for *wbII*

In order to overexpress *wbII* in *S. lividans* TK24, the *wbII* gene was amplified from the genomic DNA of *S. lividans* TK24 by PCR using primers ExpWbII-BamHI and ExpWbII-HindIII. The PCR products were digested by BamHI and HindIII, and cloned into pWHM3-*ermE*<sup>\*</sup>, a derivative of high-copy-number vector pWHM3. This contains the strong, constitutive *ermE*<sup>\*</sup> promoter, an efficient ribosomal binding site and confers resistance to thiostrepton [34]. The constructed pWHM3-*ermE*<sup>\*</sup>-*wbII* and the control empty vector pWHM3-*ermE*<sup>\*</sup> were transformed into *S. lividans* TK24 protoplasts and transformants were selected in the presence of thiostrepton at 50  $\mu$ g/ml. A method of in-frame deletion [32] was used to construct the *wbII* deletion mutant. Two 1.5 kb DNA fragments flanking the *wbII* coding region were amplified from the *S. lividans* TK24 genomic DNA by PCR using primer pairs WbII-Up1/WbII-Up2, WbII-Down1/WbII-Down2. The resulting two PCR fragments were individually digested with the corresponding restriction enzymes, and subsequently cloned into pDH5 [32] cut by HindIII and EcoRI using a triple ligation strategy, giving pDH5- $\Delta$ *wbII*. The resulting plasmid was used to delete *wbII* using the following procedure: protoplasts of *S. lividans* TK24 transformed with pDH5- $\Delta$ *wbII* were regenerated on R2YE medium without antibiotic selection at 28°C until fully sporulating. Spores were harvested and plated on R2YE medium in order to have well isolated colonies. Once sporulated these well-separated colonies were replica plated on R2YE and R2YE containing thiostrepton (50  $\mu$ g/ml) to identify the thiostrepton-sensitive colonies. The chromosomal structure of the wild-type strain and the thiostrepton-sensitive colonies was compared in the *wbII* region by PCR, using primers ExpWbII-NdeI and ExpWbII-XhoI. The near-complete deletion of *wbII* gene in *S. lividans* TK24 was verified by the size of the amplified PCR products (396 bp for wild type strain, 96 bp for mutant strain).

### Quantification of RED and ACT production

Quantification of undecylprodigiosin (RED) and actinorhodin (ACT) (both intra and extracellular) production was carried out as documented previously [31]. Briefly, 10<sup>7</sup> spores of various *Streptomyces* strains were spread on the surface of cellophane discs laid on R2YE plates and cultivated at 28°C for 72 h. In order to assay the cellular bound RED and intracellular ACT,

approximately 50 mg of mycelium (dry weight) was collected and extracted by vortexing for 30 min at 4°C in 1 ml methanol and in 1 ml KOH (1N) respectively. For RED assay, the extract was acidified to pH 2~3 by HCl and the OD<sub>530nm</sub> was determined using a spectrometer (SHIMADZU). To assay intracellular ACT, the OD<sub>640nm</sub> of the extract was measured using the same spectrometer. For extracellular ACT determination, one-quarter of an 8 cm diameter plate of the agar R2YE medium was collected, smashed and immersed in 10 ml H<sub>2</sub>O for 24 h at 4°C. The mixture was centrifuged and the resulting supernatant was transferred into a fresh tube and 10 ml KOH (1M) was added. After gentle inversion, 10 ml of HCl (3M) was added and the mixture incubated on ice for 10 min then centrifuged. The supernatant was discarded and the pellet containing ACT was re-suspended in 1 ml of KOH (1M) and the OD<sub>640nm</sub> of the resulting solution was determined. Each sample was processed in triplicate.

### Overexpression and purification of Histidine-tagged WbII in *E. coli*

The *wbII* coding sequence was amplified from the genomic DNA of *S. lividans* TK24 by PCR using primers ExpWbII-NdeI and ExpWbII-XhoI. The resulting DNA fragments were cloned into pET22b(+) cut by *NdeI* and *XhoI*, and the resultant plasmid transformed into *E. coli* BL21, a suitable host for protein expression. The resulting transformants were cultivated in LB medium supplemented with 50 µg/ml ampicillin at 37°C until OD<sub>600</sub> reached 0.6. IPTG was then added at a final concentration of 1 mM, and incubation was pursued for 6 extra hours. The cells were collected by centrifugation at 12,000 g for 10 min. Then the cell pellet was resuspended in lysis buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 500 mM NaCl) and sonicated (Hielscher Ultrasonics UP400S, 0.5 cycle and 20% amplitude) on ice until reaching complete homogeneity. After centrifugation at 14,000 rpm for 20 min, the cell extract was saved and passed through an Ni-NTA column (Cat. No. 30210; Qiagen) on a Biologic LP apparatus (Bio-Rad). The six-histidine-tagged WbII (His<sub>6</sub>-WbII) was purified to near homogeneity according to the manufacturer's instructions.

### Isolation of the putative WbII interacting targets

The putative WbII binding targets were isolated as described previously [35]. Briefly, 100~200 pmol of purified His<sub>6</sub>-WbII was incubated with 10 pmol of *S. lividans* TK24 genomic DNA for 15 min at room temperature in the binding buffer, total volume of 1 ml. A negative control reaction that contained only genomic DNA, free of WbII, was carried out in parallel. The binding reaction was fixed by addition of 1 ml of crosslinking buffer (HEPES, NaCl, EDTA, 37% formaldehyde) and incubated at 37°C for 10 min then at 4°C for 1 h. Genomic DNA was sheared to an average size of 2~3 kb by sonication for 8 sec on ice (Hielscher Ultrasonics UP400S, 0.5 cycle and 20% amplitude). The complex of His<sub>6</sub>-WbII crosslinked with fragments of genomic DNA was isolated by passing through an Ni-NTA Agarose (Cat. No. 30210; Qiagen) column. The de-crosslinking of WbII-DNA was carried out by incubation overnight with 200 mM NaCl at 65°C for 4 h and proteinase K (at a final concentration of 20 µg/ml). The de-crosslinked DNA was recovered by EtOH precipitation. The resulting DNA pool was digested by *Sau3AI*, and subsequently cloned into pUC18 cut by *BamHI*. The inserted DNA fragments were then identified by nucleotide sequencing. The promoters present on the sequenced fragments and/or its flanking regions (within 2~3 kb) were retained for further analysis.

### Electrophoretic mobility shift assay (EMSA)

The promoter regions of *wbIA*, *wbII*, *actVA3* and the intergenic region between *actII-1* and *actII-2* were amplified from *S. lividans* TK24 genomic DNA by PCR using primer pairs BS3579F/BS3579R, BS5046F/P5046R, BS5078F/BS5078R, BS82-83F/BS82-83R (Table 1),

Table 1. Synthetic oligonucleotides used in this study.

Primer	5' → 3' sequence <sup>a</sup>	Positions <sup>b</sup>	Purpose
ExpWblI-BamHI	ATAAAAGGATCCTGACGCATCGTCTCTCGCTAGC	-46 to +406	Amplification of the <i>wblI</i> coding sequence for overexpression
ExpWblI-HindIII	ATAAAAAGCTTAGAGGGTGCCCTTTCGGGTG		
WblI-Up1	ATAAAAAGCTTTTCTCTCTCCACATGGTCAG	-1500 to +80	Amplification of the 1.5 kb fragment located upstream of <i>wblI</i>
WblI-Up2	ATAAAATCTAGATCTTGGTCCCTGTCCCGC		
WblI-Down1	ATAAAATCTAGAGAATCAGGAACGCCGCC	+327 to +1860	Amplification of the 1.5 kb fragment located downstream of <i>wblI</i>
WblI-Down2	ATAAAAGAATTTCAGGACGATGACCCCGAGG		
ExpWblI-NdeI	ATAAAACATATGGTGC <sup>a</sup> TGCAACCGCCGCATTCGTC	+1 to +23	Amplification of <i>wblI</i> coding sequence for protein expression
ExpWblI-XhoI	ATAAAACTCGAGGCCGCGCCGCTATGCG	+357 to +375	
BS3579F	AGCCAGTGGCGATAAGCGTATCAATACGTCCGGCGA	-377 to +51	Amplification of the <i>wblA</i> promoter for EMSA
BS3579R	AGCCAGTGGCGATAAGATCGGTAGTCCGGCAGGC		
BS5046F	AGCCAGTGGCGATAAGCGAGTACCAGCAGGTCGTCA	-385 to +20	Amplification of the <i>wblI</i> promoter for EMSA
BS5046R	AGCCAGTGGCGATAAGCTACCTGCAGGGACGAAT		
BS5078F	AGCCAGTGGCGATAAGGTCCTCGTTCGCGTCAACAC	-395 to +8	Amplification of the <i>actVA3</i> promoter for EMSA
BS5078R	AGCCAGTGGCGATAAGGTA <sup>a</sup> CTCATCCAGCCGCCCT		
BS82-83F	AGCCAGTGGCGATAAGCGACAGTGCCTCCTCATCGT	-116 to +89 (relative to the ACTII-2 translation start as +1)	Amplification of the <i>actII-1/actII-2</i> promoter for EMSA
BS82-83R	AGCCAGTGGCGATAAGGTTCCGTCCGGTCCGGGG		
Plabel	AGCCAGTGGCGATAAG		FITC labeling
RT3579F	ATGGGCTGGGTAACCGACTG	+1 to +80	RT-PCR of <i>wblA</i>
RT3579R	GCTGCTCCCTGAACGAACAG		
RT5078F	CCATCCGTACGACGACGTGA	+357 to +474	RT-PCR of <i>actVA3</i>
RT5078R	CCGGCTGTGTGTGAGGAAGA		
RT5082F	CAGGAGCTGAAGACCGGACA	+175 to +320	RT-PCR of <i>actII-1</i>
RT5082R	ATCTCCTTGACCTGCTCCGC		
RT5083F	ATGACGCCTGGTTCGGTGT	+1027 to +1173	RT-PCR of <i>actII-2</i>
RT5083R	CTGGTCGCCGATCGTCAACAGCAT		
RT-hrdB-F	AGGACGGCGACAGCGAGTTC	+1235 to +1394	RT-PCR of <i>hrdB</i>
RT-hrdB-R	CCGAAGCCGATGGAGACG		

<sup>a</sup> Underlined nucleotides show no homology to the template; they were used for FITC labeling.

<sup>b</sup> Positions relative to the translational start site as +1.

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respectively. The resulting PCR products were 5' labeled with FITC by PCR using primer Plabel (Table 1). 50 pmol of each of the promoter regions was incubated with purified His<sub>6</sub>-WblI in various concentrations for 15 min at room temperature in binding buffer (10 mM Tris-HCl, 50 mM KCl, 1mM DTT, pH 7.5), total volume of 20 µl. For the competition assay, excess amounts of specific competitors of unlabeled *wblI* promoter or non-specific competitors of an unlabeled unrelated DNA probe were introduced. After incubation, the reaction mixtures were resolved on a 5% native polyacrylamide gel pre-run at 100 V for 30 min and run at 100 V for 90 min in a running buffer containing 45 mM Tris-HCl, pH 8.3, 45 mM boric acid, 10 mM EDTA. Visualization of the DNA signal was carried out by fluorescence imaging using a UVI Alliance 4.7 imager (UK).

### Isolation of total RNA and RT-PCR

*Streptomyces* mycelia were collected from three independent R2YE plates at various time points and total RNA isolated using an RNAPrep kit (TIANGEN, Cat. No. DP430) according to the manufacturer's instructions. Two micrograms of each RNA sample were used as a

template for the first strand cDNA synthesis for various genes using gene specific primers of RT3579R, RT5078R, RT5082R, RT5083R and RT-hrdB-R, respectively. Quantitative real-time PCR was performed in a 20  $\mu$ l reaction mixture comprising SYBR Green Real-time PCR Master Mix (Toyobo, Japan), ten percent of the cDNA synthesis reaction mixture (2  $\mu$ l), each of the gene-specific primer pairs, including RT3579F/RT3579R, RT5078F/RT5078R, RT5082F/RT5082R, RT5083F/RT5083R and RT-hrdB-F/RT-hrdB-R as internal control. Real-time PCR was run on an iCycler iQ instrument (Bio-Rad, USA.) following the manufacturer's instructions. The PCR cycling conditions were 95°C for 4 min, 35 cycles at 94°C for 30s, 62°C for 30s, 72°C for 20s and a gradient from 65°C to 95°C for 10 min under a continuous monitoring. *hrdB*, an housekeeping gene encoding the major vegetative sigma factor whose expression is constant throughout growth, was used as an internal control to normalize the relative expression of each target gene.

## Results

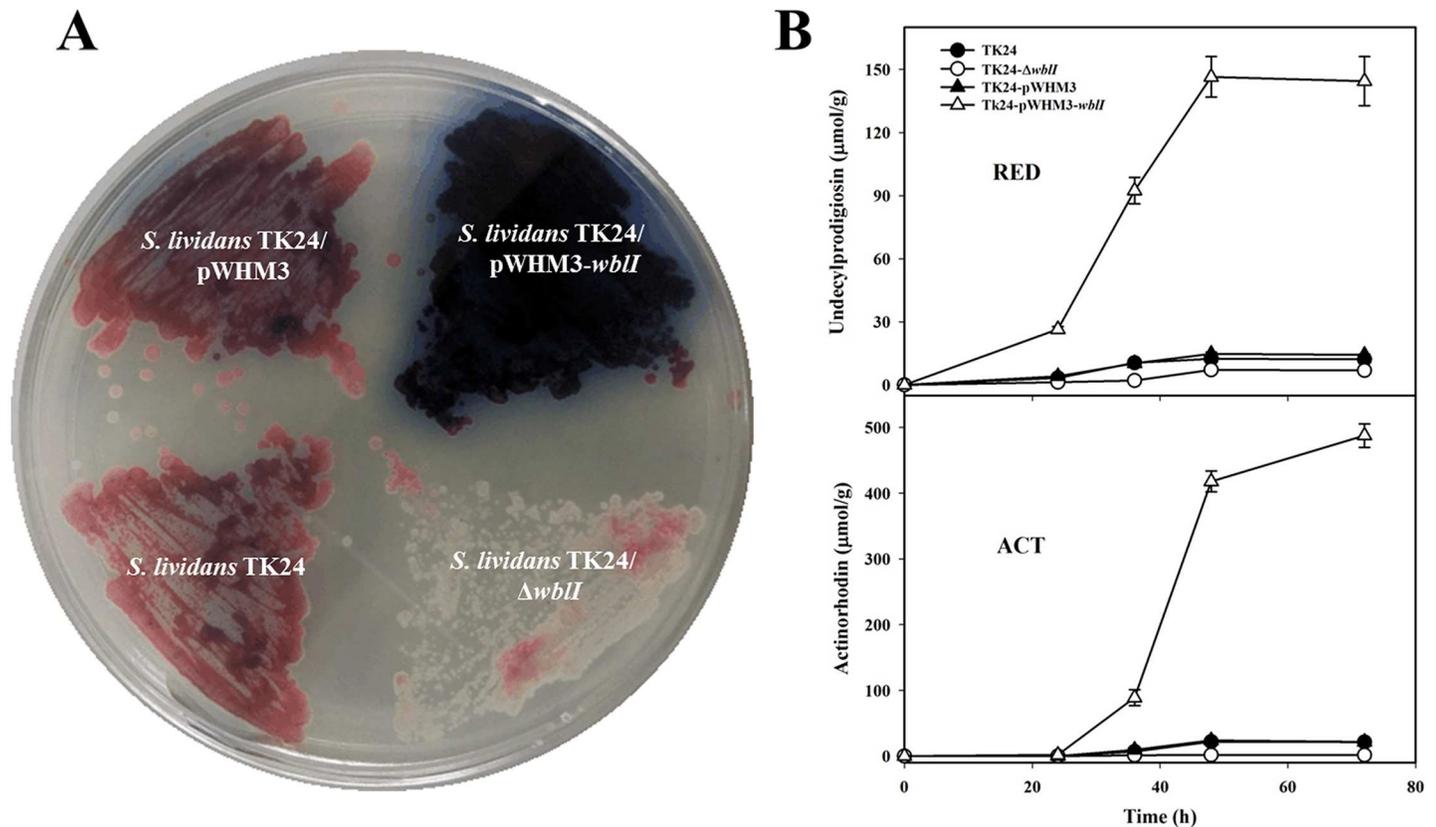
### WbII over-expression has a positive impact on RED and ACT biosynthesis

The production of RED and ACT was assayed in the original strain, *S. lividans* TK24, in the strain deleted for *wbII* and in derivatives of *S. lividans* TK24 carrying the empty plasmid pWHM3-*ermE*<sup>\*</sup> or the plasmid pWHM3-*ermE*<sup>\*</sup>-*wbII*. Results are shown in Fig 1. The over-expression of WbII, led to strong enhancement of the production of RED and ACT, the red hybrid peptide-polyketide and blue polyketide antibiotics usually weakly produced by this strain (Fig 1A) but had no impact on growth (Figure A in S1 Appendix). Quantitative analysis showed that at 72 h, *S. lividans* TK24 over-expressing *wbII* produces 10 and 23 fold more RED and ACT, respectively than the control strain (Fig 1B). Similar results were obtained when WbII of *S. coelicolor* (WbII<sup>SC</sup>) was over-expressed (data not shown). WbII and WbII<sup>SC</sup> differ by one amino acid located outside of the WhiB helix turn helix domain (Figure B in S1 Appendix). As anticipated, the deletion of *wbII* had little (or a slight negative) impact on the already weak production of these antibiotics in *S. lividans* TK24 (Fig 1). These results demonstrated the positive regulatory effect of WbII on ACT and RED biosynthesis in *S. lividans* TK24. In order to determine whether this effect was direct or indirect, attempts were made to isolate putative regulatory targets of WbII using *in vitro* formaldehyde crosslinking.

In contrast, the over-expression of WbII was shown to be correlated with the reduced synthesis of a yellow pigment (Fig 2A) thought to be the metabolic product of the *cpk* gene cluster regulated by the  $\gamma$ -butyrolactone signaling molecule SCB1 [36–38]. We noticed that the spores generated by *S. lividans* TK24/pWHM3-*ermE*<sup>\*</sup>-*wbII* remained white even upon prolonged incubation and never developed the usual grey color of the control strain *S. lividans* TK24/pWHM3 on the GYM solid medium (Fig 2B). However, spores counts with Thomas cell or spores plating in dilution yielded similar number of spores for the two strains (data not shown). This indicated that the over-expression of WbII had little if any impact on morphological differentiation.

### Isolation of the putative regulatory targets of WbII using *in vitro* formaldehyde crosslinking

*In vitro* formaldehyde cross-linking of the purified His<sub>6</sub>-WbII with its putative targets in the *S. lividans* chromosome was performed, followed by the covalent capture of WbII/DNA complex on a Ni-NTA column. After cross-linking reversal, the isolated DNA fragments were cloned into pUC18. Twenty-one clones were obtained and sequenced. Four putative target promoter



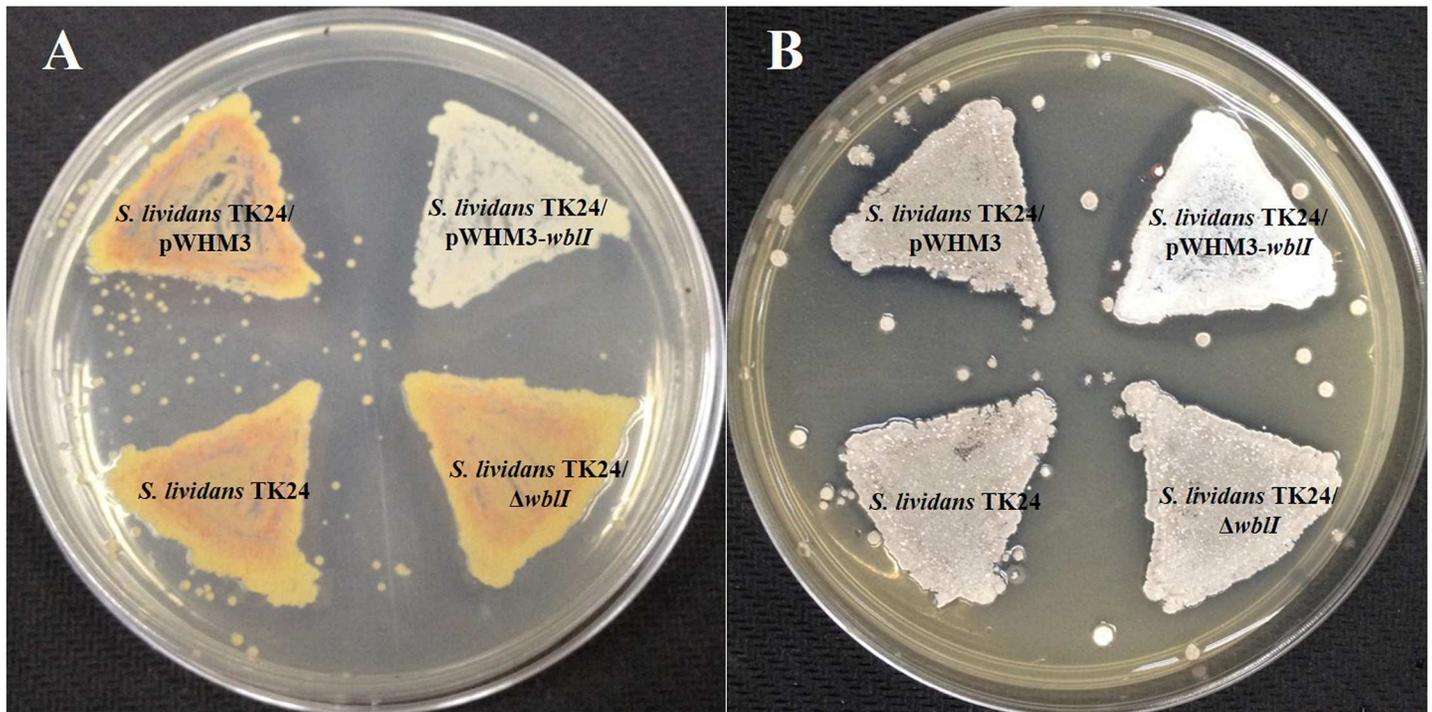
**Fig 1. Impact on secondary metabolite production by the introduction of the plasmids pWHM3, pWHM3-wbII and of the deletion of wbII ( $\Delta wbII$ ) in *S. lividans* TK24.** (A) Picture of patches grown on R2YE medium for 72 h; (B) Quantitative analysis of RED and ACT productions, all values were expressed as means  $\pm$  SD (n = 5).

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regions (absent in the negative control, see [materials and methods](#)) were identified ([Table 2](#)). Two of the targets belong to the ACT biosynthetic gene cluster. These include the region upstream of *actVA3*, a biosynthetic gene of the ACT cluster [39] and the intergenic region between two divergently located genes, *actII-1* and *actII-2*. *actII-1* encodes a TetR regulator known to repress the expression of the divergent gene *actII-2* encoding the ACT transporter [40]. The other targets of WblI were WblI itself and another WhiB-like transcriptional regulator, WblA. The latter was shown to be essential for an early stage of aerial hyphal development [23] and to have a negative impact on the oxidative stress response [25,26] and antibiotic production [24].

### WblI directly interacts with its regulatory targets in vitro

In order to confirm the direct interaction of WblI with its four putative targets, regions encompassing the promoter sequences predicted by the online Neural Network Promoter Prediction tool ([www.fruitfly.org/seq\\_tools/promoter.html](http://www.fruitfly.org/seq_tools/promoter.html)) were amplified and FITC-labeled by PCR and used as probes for EMSA. Results are shown in [Fig 3A](#). The migration of the promoter fragments of *wbII*, *wblA*, *actVA3*, as well as the intergenic region between *actII-1* and *actII-2* were found to be retarded in the presence of purified WblI in a concentration dependent manner. In all cases, addition of an excess amount of unlabeled probes led to the fading or even disappearance of the shifted bands, whereas the competition assay was largely compromised when an irrelevant DNA probe (+1 to +378 relative to the translational start codon on *wbII*



**Fig 2. Effects of *wblI* overexpression and deletion on the production of yCPK (A) and spore grey pigment (B).** Strains were cultivated on GYM medium for 36 h (A) and 72 h (B), respectively.

<https://doi.org/10.1371/journal.pone.0174781.g002>

coding sequence) was used. These results demonstrated the functionality of the purified protein as well as the specificity of the protein-DNA interactions.

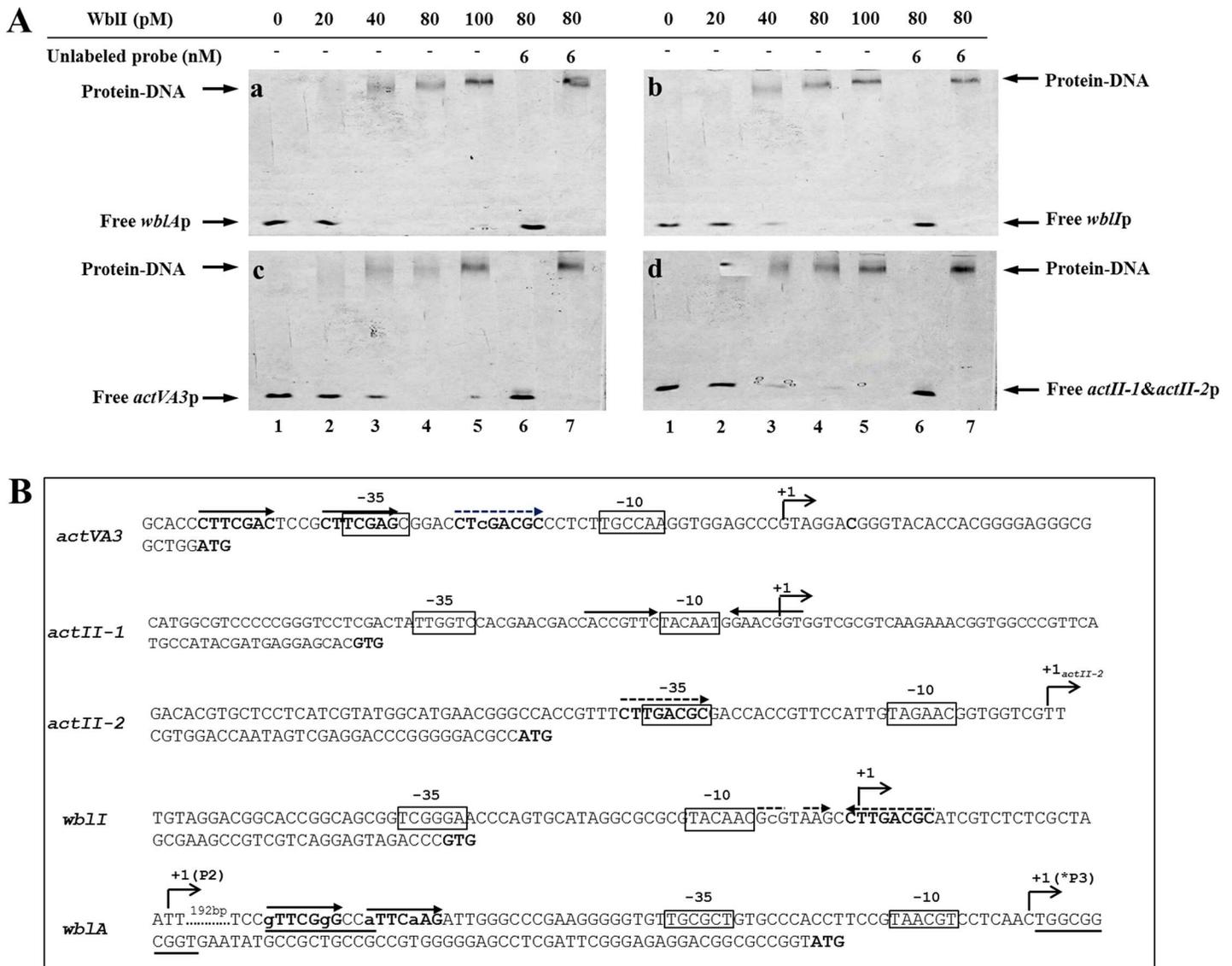
The four target promoter regions were inspected for the presence of conserved motifs (Fig 3B). Three repeats of closely related sequences CTTCGAS (S standing for G or C) or CTTGACGC were found upstream of the -35 region of *actVA3*. Their position is consistent with the activation of the expression of this gene by WblI. An perfect inverted repeat of the sequence (CACCGTTC TACAATG GAACGGTG) was found bracketing the -10 promoter region of *ActII-1* (TetR regulator) consistent with the repression of this gene by WblI. In contrast, a single repeat of the sequence (CTTGACGC) that could not constitute an operator site was found overlapping the -35 promoter region of *ActII-2* (Act transporter). An imperfect inverted repeat of the sequence CTTGACGC was found downstream of the -10 promoter sequence of *wblI*, suggesting a negative auto-regulation of WblI. Two direct repeats of a degenerated version of the sequence CTTCGAS (gTTTCGgC CC aTTCaAG) were found between the P2 and P3 promoters

**Table 2. List of putative WblI regulatory targets.**

Target gene	Protein	EMSA	In vivo test
<i>wblA</i>	WblA, putative transcriptional regulator	✓	Down
<i>wblI</i>	WblI, putative transcriptional regulator	✓	NA
<i>actVA3</i>	ActVA3, hypothetical protein of ACT biosynthetic cluster	✓	Up
<i>actII-1</i>	ActII-1, TetR family transcriptional regulator	✓	Down
<i>actII-2</i>	ActII-2, probable ACT transporter	✓	Up

✓: promoter regions shifted up by WblI in EMSA; Up or Down: transcription up or down regulated by overexpressed WblI in RT-PCR; NA: not applicable.

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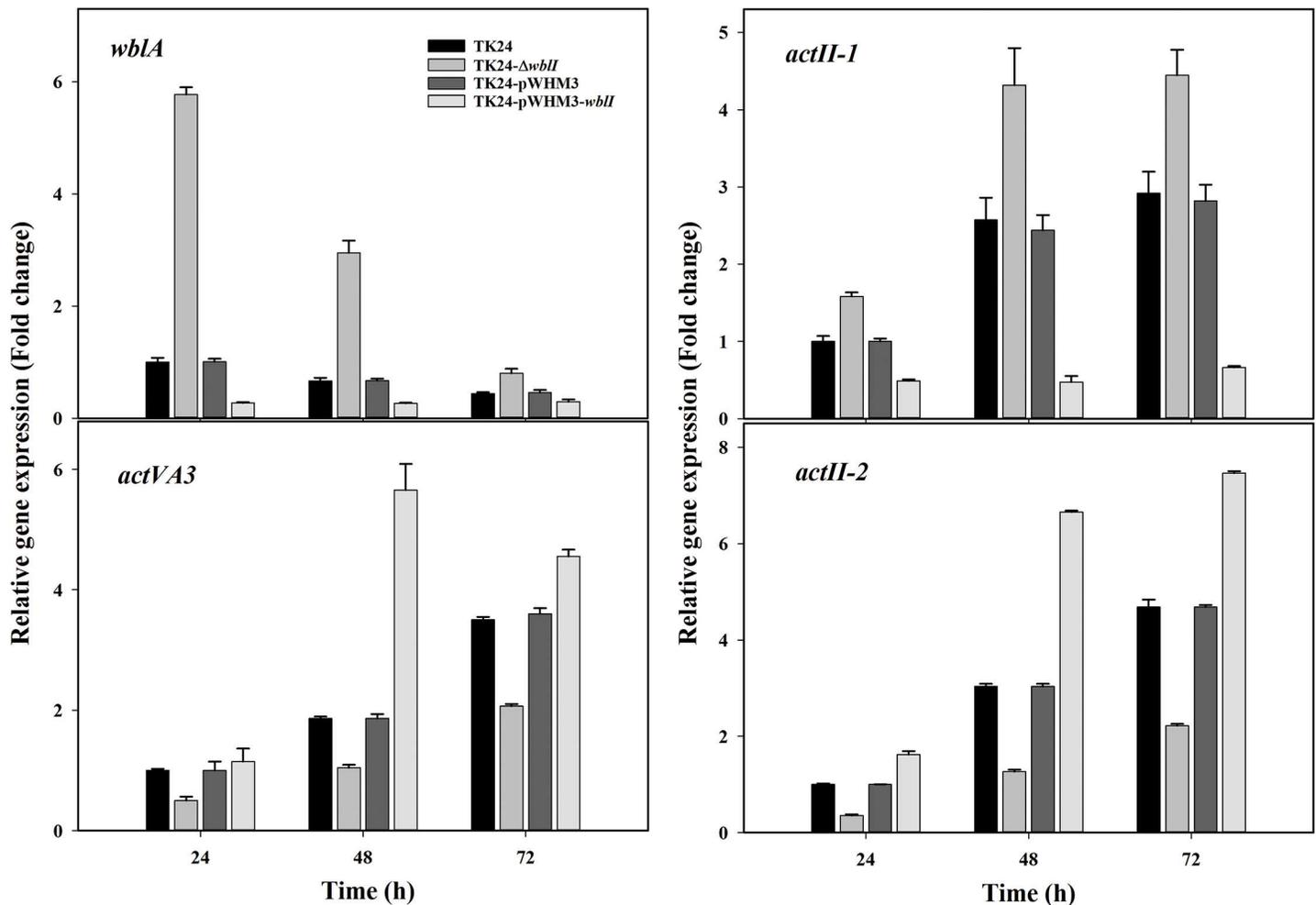
**Fig 3.** (A) Electrophoretic mobility shift assay of purified WblI with its putative regulatory promoter targets: *wblA* (a), *wblI* (b), *actVA3* (c) and the intergenic region between *actII-1* and *actII-2* (d). In all cases, 50 pmol of FITC-labeled probe was used. The specific (unlabeled target promoters) and non-specific (irrelevant DNA) competitors were introduced in lane 6 and lane 7, respectively. Arrows indicate the positions of DNA-protein complexes and free DNA. (B) Sequence of the promoter regions of *actVA3*, *actII-1*, *actII-2*, *wblI*, *wblA*. The transcriptional sites are indicated by +1 with bent arrows. Putative -10 and -35 regions are boxed. Translational start codons are in bold. The two related direct or inverted repeats of the sequences CTTTCGAS or CTTGACGC thought to constitute WblI operator sites are shown as plain or dotted arrows above the sequence line. AdpA binding motifs are underlined.

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of WblA in overlap with a known negative regulatory site for AdpA [41]. Even though these putative binding sites should be confirmed by foot printing experiments, their localization fits the observed regulatory features of these genes by WblI.

### Assessment of in vivo regulatory effects of WblI using quantitative RT-PCR

In order to assess *in vivo*, the impact of WblI on the expression of the target genes validated by EMSA, mRNA was prepared from the wild type strain of *S. lividans* TK24 carrying the empty vector or carrying the *wblI* over-expression plasmid or deleted for *wblI*, at 24, 48 and 72 h. The



**Fig 4. Quantitative analysis of the transcriptional levels of WblI regulatory targets by RT-PCR in the wild type strain of *S. lividans* TK24, in the strain deleted for *wblI* and in the strains carrying *wblI* overexpressing plasmid or the empty plasmid at 24, 48 and 72 h. All values were expressed as means  $\pm$  SD (n = 5).**

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expression levels of *wblA*, *actVA3*, *actII-1* and *actII-2* were examined by qRT-PCR. Results are shown in Fig 4. In the wild type strain, the expression of genes of the ACT cluster (*actVA3*, *actII-1* and *actII-2*) was enhanced at 48 h, a time point where ACT was detectable. At the three time points tested, the over-expression of WblI was correlated with the increased expression of the genes encoding the hypothetical protein ActVA3 and the ACT transporter ActII-2, while transcription of the divergently located TetR regulator ActII-1 and of WblA was reduced. Consistently, the deletion of *wblI*, at all three time points, was correlated with reduced expression of *actVA3* and of *actII-2* and with enhanced expression of *actII-1* and *wblA*. Taken together with the above EMSA data, these results demonstrated that WblI positively regulates the transcription of *actVA3* while negatively controlling that of *actII-1* and *wblA* via direct interaction with their promoter regions.

### Overexpression of *wblI* enhances ACT export

Since the TetR regulator ActII-1 that regulates negatively ActII-2, the ACT export system, was proposed as a WblI regulatory target, the ratio between extracellular (exported) and

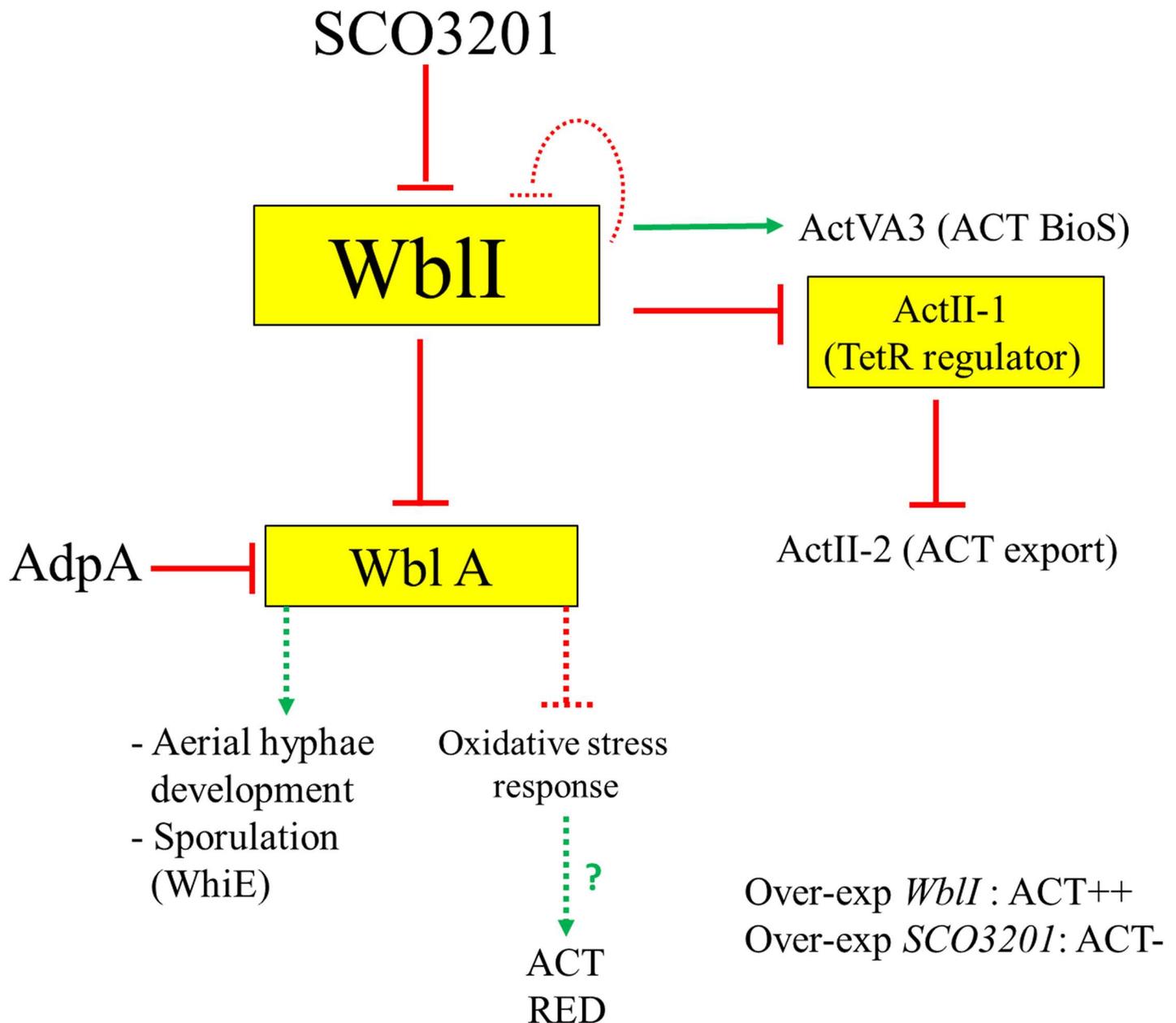
intracellular ACT was determined in strains of *S. lividans* TK24 carrying the empty vector (pWHM3-*ermE*<sup>\*</sup>) or the *wbII* overexpression plasmid (pWHM3-*ermE*<sup>\*</sup>-*wbII*). This ratio was found to be 1.3 fold higher in the WbII overproducing strain than in the control strain indicating that WbII had a positive impact on ACT export (Table A in [S1 Appendix](#)).

## Discussion

The WhiB-like transcriptional regulator [35], WbII (SCO5046), studied in this issue was first identified as a target of the transcriptional regulator of the TetR-family (SCO3201). The overexpression of SCO3201 was shown to lead to strong repression of both antibiotic production and sporulation in *S. coelicolor* [31]. However, since the disruption of *SCO3201* did not lead to any obvious phenotype, it was proposed that SCO3201 “illegitimately” governs the expression of target genes normally under the control of other TetR regulators truly involved in the regulation of the differentiation process. It was thus inferred that the analysis of the “SCO3201” targets might lead to the identification of new players in the complex regulation of the differentiation process in *S. coelicolor*. Indeed, the search for SCO3201 targets allowed the identification of genes already known to be involved in the regulation of the differentiation process, validating this approach, as well as of new players including WbII (SCO5046).

In the present paper, we report the consequences of the over-expression of *wbII* on secondary metabolites production of *S. lividans* and the characterization of four of the putative regulatory targets of this regulator. The latter were identified using formaldehyde crosslinking followed by covalent capture of WbII/DNA complex on a Ni-NTA column, and were subsequently confirmed as direct targets by EMSA (Table 2, Fig 3) and qRT-PCR (Fig 4). Furthermore, an inspection of the promoter regions of the targets genes revealed some putative similar regulatory sequences. Altogether these investigations indicated that WbII represses its own transcription as well as that of the TetR regulator *actII-1* that represses the expression of the divergent gene *actII-2* encoding the ACT transporter [40]. qRT-PCR analysis confirmed that the transcription of *actII-1* was reduced whereas that of *actII-2* was enhanced in the WbII over-expressing strain. A putative WbII binding sequence was found to bracket the -10 region of *actII-1* suggesting repression of the expression of the latter by WbII. Consistently an enhanced ACT export was correlated with WbII over-expression. This enhanced export ought to be correlated with an enhanced ACT biosynthesis. The latter might be achieved *via* the positive effect that WbII exerts on the expression of *actVA3*, the third gene of a succession of six genes of the ActVA3 region (Fig 4). Indeed, three repeats of the putative WbII operator sequence were found upstream of the -35 promoter region of *actVA3*, in a position expected for an activator site. The function of ActVA3 is unknown but we propose that it might constitute a bottleneck in the ACT biosynthetic pathway. In the *wbII* over-expressing strain, its enhanced biosynthesis would thus contribute to observed enhanced ACT biosynthesis.

In contrast, the over-production of WbII was correlated with a reduction of the biosynthesis of the yellow mycelial pigment and of the grey spore pigment encoded by the CPK [42] and *whiE* loci, respectively (Fig 2). The synthesis of grey spore pigment, regarded as a sign of completion of spore maturation, is directed by the *whiE* locus composed of an operon of seven genes and a gene transcribed in the opposite direction [43,44]. The transcription of genes of this locus have been shown to rely on the presence on six known *whi* genes (including *whiB*), which are required for sporulation septum formation [44]. However, none of the *cpk* or *whiE* genes were identified as putative WbII target genes in our formaldehyde cross linking experiments (Table 2). This suggests that the negative impact that WbII has on these genes may be indirect. ACT,  $\gamma$ CPK and WhiE are all polyketides so they are likely to compete for a common precursor, acetyl-CoA. Enhanced ACT biosynthesis would thus lead to decreased acetyl-CoA



**Fig 5. Schematic representation of the WblI and WblA regulatory network.** Transcriptional regulators are boxed. Continuous and dotted lines represent proven direct or indirect regulatory interactions, respectively. Flat-headed and pointed arrows indicate repression and activation, respectively.

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availability and thus reduced  $\gamma$ CPK and grey pigment synthesis. This view is supported by the fact that the *wblI*-deletion mutant did not have any obvious phenotype in relation to yellow pigment production or spore pigmentation (Fig 2).

Finally and most importantly, our RT-PCR experiments revealed reduced expression of the *wblA* gene in the WblI over-expressing strain (Fig 4). In *S. coelicolor* the expression of WblA was shown to be under the negative control of AdpA [41], a regulator playing a positive role in aerial mycelium development and ACT production. The promoter region of WblA is very complex comprising 3 promoters and 6 putative AdpA binding motifs [41]. Interestingly, two

direct repeats of a degenerated version of the putative WblI operator sequence CTTCGAS (gTTTCGgG CC aTTCaAG) was noted between the P2 and P3 promoters of WblA (gTTTCGgG CC aTTCaAG) in overlap of the binding site of AdpA (Fig 3B). This and our *in vivo* results suggested a negative regulation of WblA by WblI. WblA was shown to act as a negative regulator of ACT biosynthesis [24,41]. Consequently the repression by WblI, of WblA expression is consistent with enhanced ACT biosynthesis.

In *Mycobacteria tuberculosis*, Wbl proteins were shown to act as redox-sensing factors *via* their Fe-S clusters [45] and to trigger specific adaptive responses *via* their transcriptional regulator activity. The expression of the seven Wbl proteins (Whib 1–7) of this species was shown to be induced by various oxidative stresses [46]. Interestingly, in the WblA mutant of *S. coelicolor*, the oxidative stress response as well as RED and ACT biosynthesis were up-regulated [24–26]. This indicated that oxidative stress might be higher in this strain than in the original strain and suggested that oxidative stress might constitute an important signal to trigger antibiotic biosynthesis.

In summary, we provide in Fig 5 a schematic representation of the demonstrated WblI and WblA regulatory interactions. The strong enhancement that WblI over-expression has on ACT biosynthesis might result from the direct activation of ACT biosynthesis and export as well as to the repression of the expression of WblA, a likely indirect, repressor of ACT biosynthesis.

## Supporting information

**S1 Appendix.** (includes Table A, Figure A and Figure B).  
(DOCX)

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**Validation:** DLX MJV.

**Visualization:** DLX MJV.

**Writing – original draft:** DLX.

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