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Defining the OrrA regulon and its role in development and antibiotic production in *Streptomyces venezuelae* NRRL B-65442

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1.3 Keywords

Antibiotics, *Streptomyces*, chloramphenicol, WhiB-like proteins, gene regulation

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OrrA ChIP-seq data accession number = GSE300268

OrrA TMT proteomics data are available via ProteomeXchange with identifier PXD064843

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2. Abstract

Streptomyces bacteria have complex life cycles involving hyphal growth, sporulation, and the production of diverse specialised metabolites, including antibiotics. In this study, we investigated the role of the highly conserved orphan response regulator OrrA in *Streptomyces venezuelae* NRRL B-65442. We show that *S. venezuelae* Δ*orrA* mutants are defective in sporulation and, using ChIP-seq, identify five OrrA binding sites *in vivo*. Tandem-mass-tag proteomics revealed that OrrA directly activates two of these putative target genes, *wblA* and *vnz_04640*, a finding consistent with previous work on OrrA in the distantly related *S. coelicolor*. We also demonstrate that deleting *wblA* blocks sporulation and that overexpressing *wblA* restores sporulation in the Δ*orrA* mutant. Additionally, chloramphenicol biosynthesis is upregulated in both the Δ*orrA* and Δ*wblA* mutants compared with the wild type. Taken together, these results indicate that the primary function of OrrA is to regulate WblA production, and that reduced intracellular WblA levels underlie the phenotypes observed in the Δ*orrA* mutant.

3. Data summary

The authors confirm all supporting data, code and protocols have been provided within the article, through supplementary data files and via public databases.

4. Introduction

The genus *Streptomyces* comprises more than 1100 verified species and is the type genus for the ancient bacterial phylum Actinomycetota (1). *Streptomyces* species are studied for their complex development and their prolific production of specialised metabolites, which form the basis of ~55% of clinically used antibiotics (2). The *Streptomyces* life cycle begins with spore germination and outgrowth into a branching, vegetative substrate mycelium which, upon nutritional stress, gives rise to reproductive aerial hyphae that undergo rapid DNA replication and cell division to form chains of unigenomic spores (3). Prior to genome sequencing, classical genetics identified many genes that are involved in development, and these were classified as either *bld* (bald) genes, that are required for aerial hyphae formation, or *whi* (white) genes that are required for sporulation (1). The *bld* mutants form colonies that do not have 'hairy' aerial mycelium and thus appear bald when growing on solid agar while the *whi* mutants form white colonies that lack the WhiE spore pigment, either because the aerial hyphae do not undergo cell division to form spores or because the spores do not mature (3).

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Two-component systems (TCS) as well as orphan response regulators (RRs) and sensor kinases (SKs) have been heavily implicated in the regulation of the *Streptomyces* life cycle and their specialised metabolism (4,5). This includes the RR MtrA which is a master regulator of specialised metabolism in *Streptomyces* species (6–9). MtrA is part of a conserved three-component system that includes its cognate SK MtrB and an accessory lipoprotein LpqB (10). Recent work reported that MtrAB senses osmotic stress in *S. venezuelae* NRRL B-65442 and activates production of the compatible solute ectoine and triggers entry into sporulation (11). An orphan RR designated OrrA (12) is encoded within the same region and the *orrA* gene (*sco3008*) is separated from the *mtrAB-lpqB* operon (*sco3013-11*). Deletion of *orrA* in *Streptomyces coelicolor* M145 resulted in a bald mutant that cannot form aerial hyphae or spores, even after prolonged incubation (13). Only two OrrA target genes were identified in *S. coelicolor* using ChIP-seq, namely *sco1375*, which encodes a small protein of unknown function, and *wblA* which encodes a member of the WhiB-like family of 4Fe-4S cluster containing transcription factors (14–16). RNA-seq analysis showed that OrrA is required to activate the expression of *sco1375* and *wblA* and the *S. coelicolor* Δ*wblA* and Δ*orrA* mutants share very similar phenotypes. However, over-expression of either gene did not fully complement the *S. coelicolor* Δ*orrA* mutant.

In this work we show that OrrA activates the production of WblA and the SCO1375 homologue Vnz_04640 in the model organism *S. venezuelae* NRRL B-65442, which is distantly related to *S. coelicolor*. *S. venezuelae* NRRL B-65442 forms green spores and has emerged as a model organism because of its genetic tractability, rapid growth and the ability to complete its life cycle to sporulation in liquid growth medium (17,18). Three additional putative OrrA target promoters were identified here using ChIP-seq and we used a DNA footprinting technique called ReDCaT SPR to identify OrrA binding sites at four of these five promoters. However, of the five putative OrrA target genes identified, proteomics analysis showed that only two of the gene products were significantly affected in abundance by loss of OrrA, namely WblA and Vnz_04640. *S. venezuelae* Δ*orrA* and Δ*wblA* mutants are both defective in sporulation and over produce the antibiotic chloramphenicol suggesting the phenotypes observed in the Δ*orrA* strain are the result of a reduction in WblA abundance. Consistent with this, over-expression of *wblA* in the Δ*orrA* mutant restored sporulation. We conclude that OrrA targets and function are conserved between *S. coelicolor* M145 and *S. venezuelae* NRRL B-65442, namely to control the expression of *wblA*, which encodes a key regulator of development and a global repressor of antibiotic biosynthesis (19).

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5. Methods

Bacterial strains. Wild-type *Streptomyces venezuelae* NRRL B-65542 and isogenic mutant strains were grown in MYM (Maltose + Yeast Extract + Malt Extract) liquid medium and on MYM agar. *Escherichia coli* strains were grown in LB (Lennox Broth) and on LB agar. Media recipes plus written and video protocols for culturing *Streptomyces* bacteria, making spore preparation and inter-species conjugation can be found at http://actinobase.org and in the *Streptomyces* Practical Genetics manual which is free to download here: https://streptomyces.org.uk/PracticalStreptomycesGenetics.pdf (20). All the bacterial strains, plasmids and primers used in this study are listed in Tables S1-S3.

CRISPR/Cas9-mediated genome editing. The *orrA* gene was deleted using pCRISPomyces-2 following the published protocol (21) using gene-specific guide RNAs and repair templates (see Table S3 for primers). A detailed protocol is also available at http://actinobase.org (22).

Chloramphenicol Analysis. Chloramphenicol was measured according to Som et al., 2017 (6). Strains were grown in biological triplicates in 30ml MYM liquid medium for 24 hours. An aliquot of 750 µL was taken from each culture and 750 µL of ethyl acetate was added. After shaking for 10 min, the extracts were centrifuged, and the organic phase (500 µL) was transferred to a 1.5 mL glass vial. The solution was concentrated under reduced pressure and the residual extract dissolved in 500 µL of a mixture of methanol:water (2:3). For quantification 40 µL of each sample was subjected to analytical HPLC. Analysis was performed on an Agilent 1290 Infinity II LC System (Agilent Technologies). A Gemini® 3.0 µm NX-C18 150 x 4.6 mm column was used. Chloramphenicol was eluted using mobile phase A (water containing 0.1% formic acid) and mobile phase B (100% methanol) using the following multistep gradient at a flow rate 800 µL min^{-1}: 0 min, 10%B; 14 min, 100%B; 18 min, 100%B; 18.5 min, 10%B; 19.5 min, 10%B. Stock solutions of chloramphenicol (Sigma Aldrich) for calibration were prepared between a range of 0.01 µg mL^{-1} and 0.1 mg mL^{-1}. Generation of the calibration curve and quantification of chloramphenicol was accomplished by integration of the chloramphenicol signal at 273 nm.

Chromatin Immunoprecipitation Sequencing (ChIP-seq). *S. venezuelae* wild-type and Δ*orrA* complemented with pSS170 + *orrA*-N-FLAG (Tables S1 and S2) were inoculated from

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single colonies onto sterile cellophane disks placed on MYM agar and incubated at 30°C for 18 and 48 hours. Under sterile conditions, lawns were scraped into 50 mL tubes, and cross-linking was performed by adding 10 mL of 1% (v/v) formaldehyde in PBS for 30 minutes at 30°C with gentle shaking (90 rpm). Cross-linking was quenched by replacing the formaldehyde with 10 mL of 0.5 M glycine and incubating for 5 minutes at room temperature. Following glycine removal, the biomass was washed twice with 25 mL of ice-cold PBS (pH 7.4) and flash frozen at –80 °C. Unless otherwise stated, all buffers used during extraction contained EDTA-free protease inhibitors (2 tablets per 10 mL). Cell lysis was performed by resuspending pellets in 2 mL of lysis buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mg/mL lysozyme) and incubating at 37 °C for 30 minutes. DNA fragmentation was initiated by adding 1 mL of immunoprecipitation (IP) buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100) and mixing by pipetting. Samples were sonicated on ice (20 cycles at 50 Hz, 10 second pulse with 1-minute rest between pulses). Fragmentation efficiency was checked by phenol–chloroform extraction of 25 µL crude lysate mixed with 75 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), followed by RNase A treatment (2 µL of 1 mg/mL) and agarose gel electrophoresis. Ideal fragment sizes ranged between 200–1000 bp. The remaining lysate was cleared by centrifugation at 1968xg for 15 minutes at 4 °C. Anti-FLAG M2 agarose beads (60 µL per sample) were pre-washed twice in 2.5 mL of 0.5× IP buffer and 60 µL was incubated with the cleared lysate overnight at 4°C on a vertical rotor. Beads were subsequently washed four times with 0.5× IP buffer (10 minutes per wash at 4 °C). Bound DNA was eluted by adding 100 µL elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and incubating at 65 °C overnight. A second elution step with 50 µL elution buffer was performed for 5 minutes at 65 °C. Combined eluates were treated with 2 µL proteinase K (20 mg/mL) at 55 °C for 2 hours, followed by phenol–chloroform extraction and purification using a QIAquick column (Qiagen). DNA was eluted in 50 µL EB buffer (10 mM Tris-HCl, pH 8.5); 5 µL was retained for quantification using Nanodrop and Qubit, and the remainder stored for sequencing. Samples were sent to Genewiz® for paired end 150bp Illumina sequencing, generating ~6 Gb of raw data at ~20 million read pairs per sample. Sequencing data were processed as described previously (23).

Reuseable DNA Capture followed by Surface Plasmon Resonance (ReDCaT SPR). The double stranded oligonucleotide probes used for the *in vitro* DNA binding studies are shown in Table S4. ReDCaT SPR was performed as described previously (24) using the purified DNA binding domain of *S. venezuelae* OrrA.

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Sample Preparation for Tandem Mass Tag (TMT) proteomics. *S. venezuelae* strains were streaked from single colonies onto MYM (pH 7.3) agar plates on top of sterile cellophane disks and incubated at 30 °C to the desired time points. The cellophane disks were then removed, and the mycelium was scraped into 50 mL tubes containing 10 mL cell lysis buffer (2 % SDS, 50 mM TEAB Buffer pH 8.0, 150 mM NaCl and EDTA-free protease inhibitor tablets, supplied by Roche). Samples were vortex vigorously to disrupt the mycelial mass and minimize clumping, then boiled for 10 minutes. Following boiling, samples were then sonicated using four 20 second pulses at 10 microns, with 1-minute intervals between each cycle. Debris was removed by centrifugation at 4,000 x g for 30 min at room temperature. The clarified supernatant was transferred to a fresh tube, and protein concentration measured using the Qubit Protein Assay. The volume containing 1 mg protein was aliquoted to a 15 mL Falcon tube. Proteins were precipitated with chloroform/methanol (25). Samples were processed by the John Innes Centre proteomics platform, as described previously (26). Detailed proteomic methods are provided in the supplementary information.

Cryo-Scanning Electron Microscopy Conducted by the Bioimaging facility at the John Innes Centre by Rhea Stringer. Samples were mounted on an aluminium stub using Tissue-TekR (BDH Laboratory Supplies, Poole, England). The stub was immediately plunged into liquid nitrogen slush (approximately −210 °C) to cryo-preserve the material. The frozen sample was then transferred to the cryo-stage of an ALTO 2500 cryo-transfer system (Gatan, Oxford, England) attached to an FEI Nova NanoSEM 450 (FEI, Eindhoven, The Netherlands). Surface frost was sublimated at −95 °C for 4 minutes, after which the sample was sputter-coated with platinum for 120 seconds at 10 mA, at a temperature below −110 °C. The sample was then transferred to the main chamber of the microscope, where the cryo-stage was maintained at approximately −125 °C. Imaging was performed at 3 kV, and digital TIFF files were recorded.

Phylogenetics. To generate the phylogenetic tree, all Actinomycetota reference genomes with a completeness level of “chromosome” or better were downloaded from the NCBI database and the RpoB and OrrA protein sequences were identified using reciprocal BLAST searching with the *Streptomyces venezuelae* NRRL B-65442 (GCF_001886595.1) gene used as the query. All resulting RpoB sequences were aligned using MAFFT (mafft --auto input.faa >

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output.fasta) (27), the resulting alignment was imported into the Galaxy EU server (28) and FastTree was used with default settings plus LG+CAT model selected to generate a maximum likelihood phylogenetic tree. Subsequently a presence/absence of OrrA, WblA and AdpA was annotated using iTol (29).

6. Results and Discussion

***S. venezuelae* Δ*orrA* is defective in sporulation.** Cas9-mediated genome editing was used to make an in-frame deletion of the *orrA* gene in *S. venezuelae* NRRL B-65442. After two days growth on MYM agar the wild-type strain forms its characteristic, green-pigmented spores while the Δ*orrA* mutant does not sporulate (Figure 1), which is consistent with the phenotype reported for *S. coelicolor* Δ*orrA* (13). This phenotype is maintained if the colonies are restreaked within three days of growth on MYM agar or used within three days to inoculate 2xYT broth to grow liquid cultures for glycerol freezer stocks. However, prolonged growth (≥5 days) results in heterogeneous colonies, some of which form aerial mycelium and spores (Figure S1).

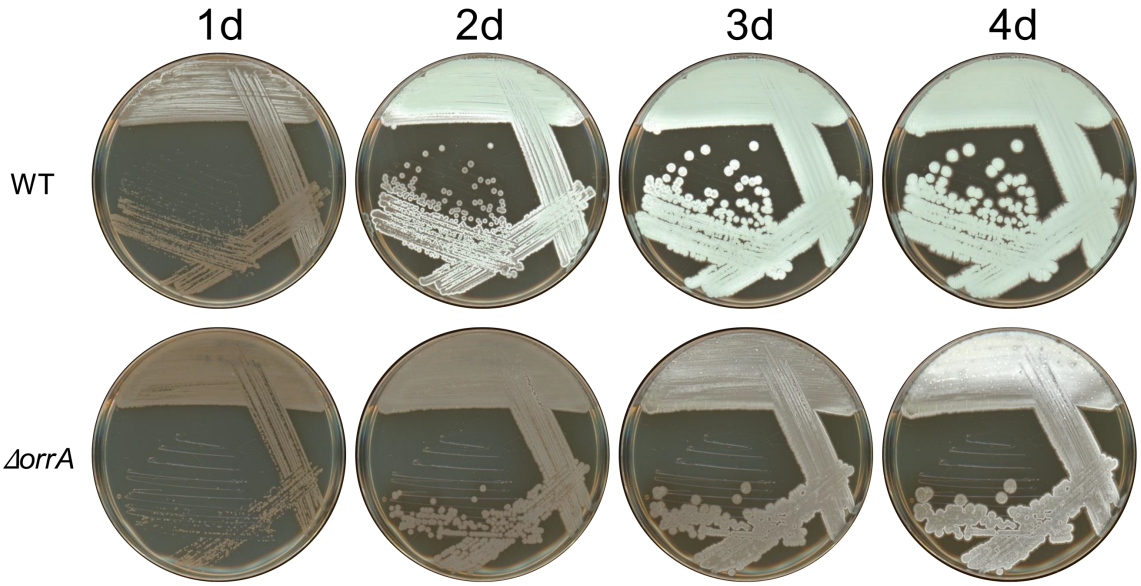


Figure 1. *S. venezuelae* Δ*orrA* is unable to sporulate. Top plates show the *S. venezuelae* wild-type (WT) growing on MYM agar for 1, 2, 3 and 4 days as indicated. Brown colouring indicates vegetative hyphal growth, white colouring indicates aerial hyphae formation and green colouring indicates sporulation because the mature spores are green. Bottom plates show cultures of the isogenic Δ*orrA* mutant grown for the same incubation times as indicated.

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Vegetative colonies are stable, but emergent aerial hyphae yield genetically and phenotypically unstable mutants (Figure S1).

Identification of OrrA target genes in *S. venezuelae.* To identify OrrA binding sites on the *S. venezuelae* genome the Δ*orrA* mutant was complemented by integrating a construct encoding OrrA with an N-terminal 3xFLAG tag *in trans* under the control of its native promoter. ChIP-seq analysis was performed in triplicate using this FLAG-tagged strain and wild-type *S. venezuelae* was used as a negative control. The sequencing data were analysed as described previously (23) and led to the identification of five promoters that were enriched in the FLAG-tagged strain relative to the control (Figure S2), including the *wblA* promoter (Figure 2) and the *vnz_04640* promoter (Figure S3).

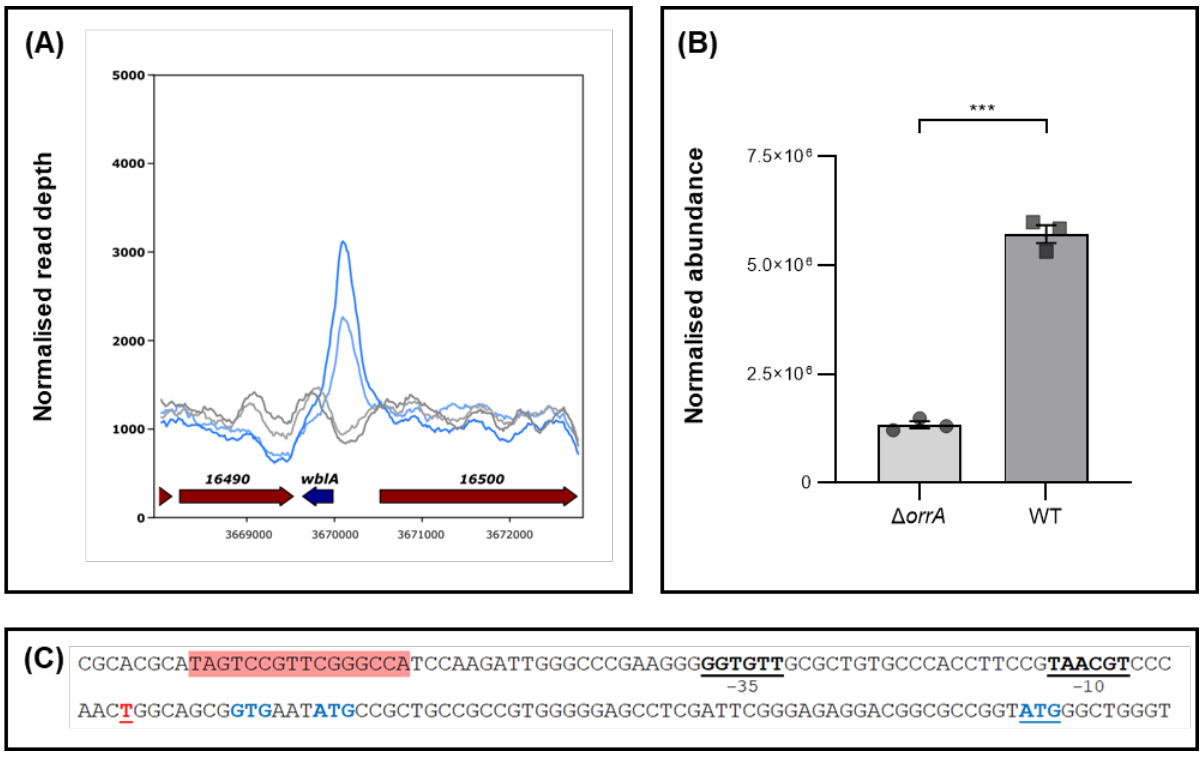


Figure 2. OrrA activates the production of WblA in *Streptomyces venezuelae.* (A). Normalised ChIP-seq read depth, blue traces show the OrrA-FLAG strain (n=2) and black traces show the wild-type control (n=2). (B). TMT proteomics data (n=3) showing the normalized peptide abundance of WblA in the *S. venezuelae* Δ*orrA* and wild-type strains at 18 hours growth on MYM medium. Individual replicate abundances are shown. *** = BH-adjusted *p*-value < 1 x 10^{-7} (background-based *t*-test). (C). The *wblA* promoter with OrrA consensus site highlighted in red, the RNA polymerase -35 and -10 sites underlined in black boldface, and the transcription start site (TSS) underlined in red boldface. There are three

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possible WblA translational start codons that are in frame, all marked in blue with the annotated start codon underlined. The same data are shown for OrrA target gene *vnz_04640* in Figure S3.

Four of these five promoters were used to measure OrrA binding *in vitro* using a DNA footprinting technique known as Reuseable DNA capture surface plasmon resonance, or ReDCaT SPR (Figures 3 and S4). ReDCaT SPR employs immobilised double-stranded oligonucleotide probes that span the promoter of interest. These probes are covalently attached to an SPR chip and subsequently challenged with the purified protein of interest (30). The full length OrrA protein could not be produced in soluble form in *E. coli*, so we used the purified DNA binding domain of OrrA for these experiments. This purified protein bound specifically to single probes at 4 of the 5 target promoters (Table 1). The *vnz_19230* promoter was not tested in these experiments because the ChIP-seq peak was overlooked in the initial analysis of the ChIP-seq data. It was identified as an OrrA target after scanning the *S. venezuelae* genome with the experimentally verified OrrA consensus binding sequence.

Target	**Function**	**TMT data Δ*orrA*/ WT**	**TMT P-value**	**OrrA binding site identified by ReDCaT SPR**	**Distance from TSS**
vnz_04640	Conserved hypothetical	0.175	4.39×10^{-8}	TAGCCCACTGGGACCA	-72
vnz_16110 (putative operon)	ABC binding protein	0.861	0.95 *	TAGTCAAATTTGACCA	N/D

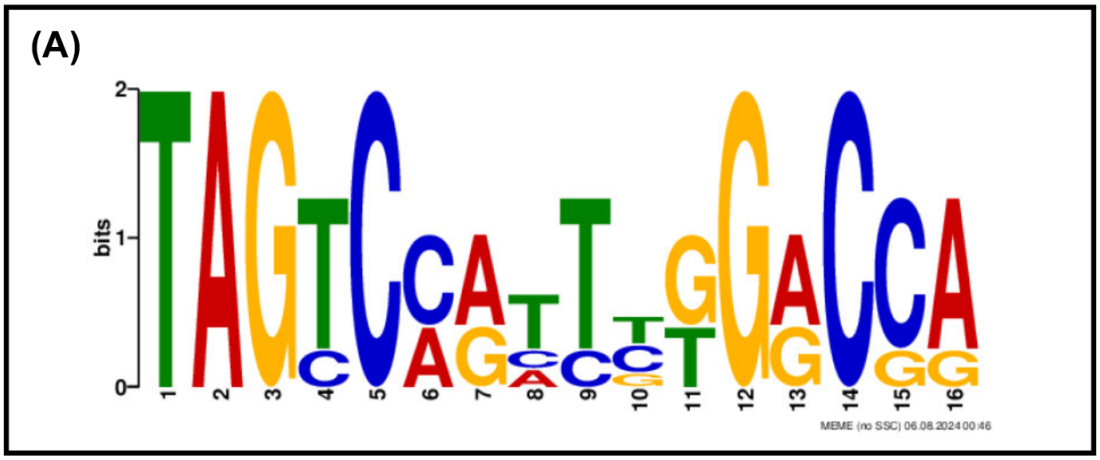
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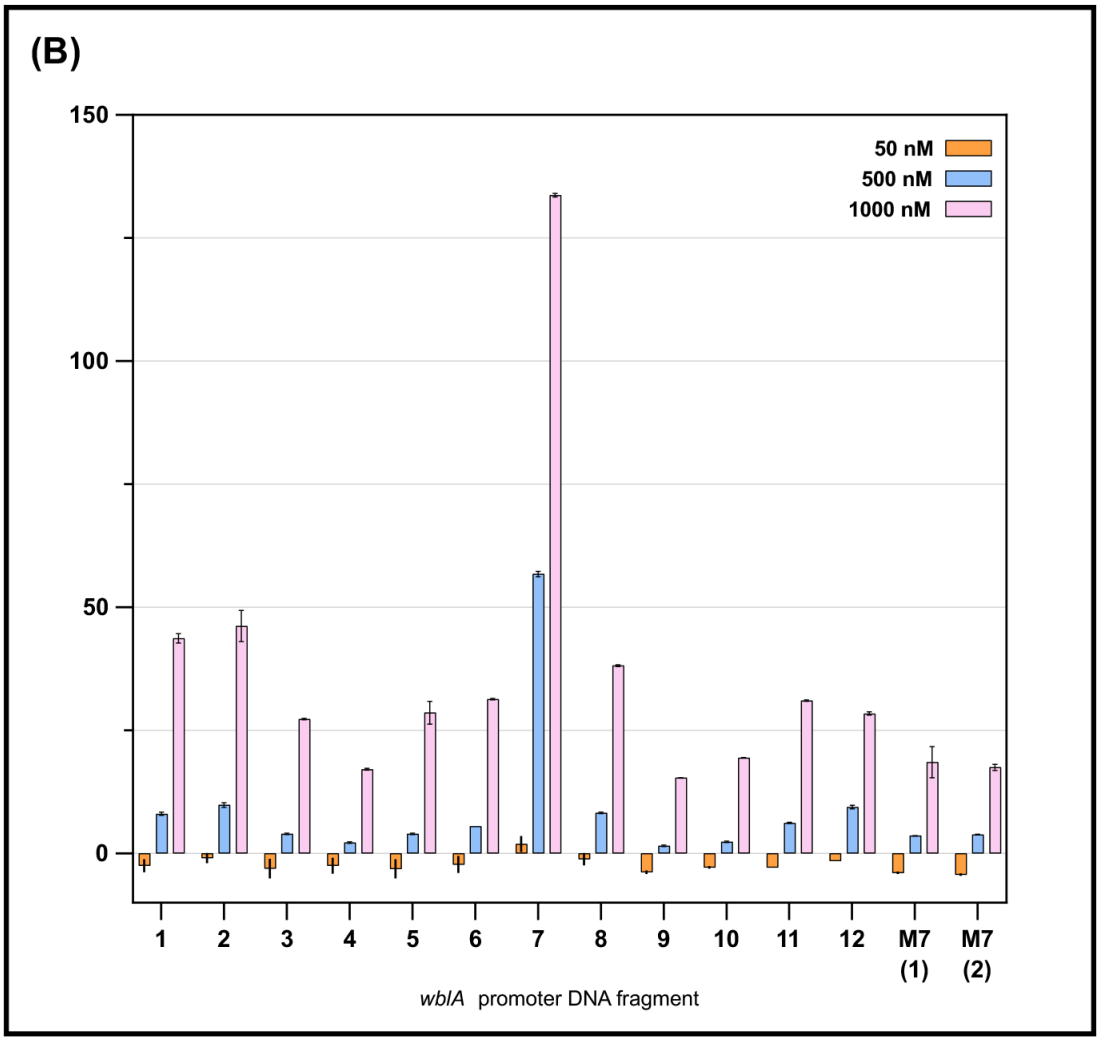
vnz_16115	PadR-family regulator	1.087	1 *	TAGTCAAATTTGACCA	-2
vnz_16495 (*wblA*)	WhiB-like regulator	0.224	4.68 x10^{-9}	TAGTCCGTTCGGGCCA	-63
vnz_19230	Hypothetical	N/D	N/D	Not tested	

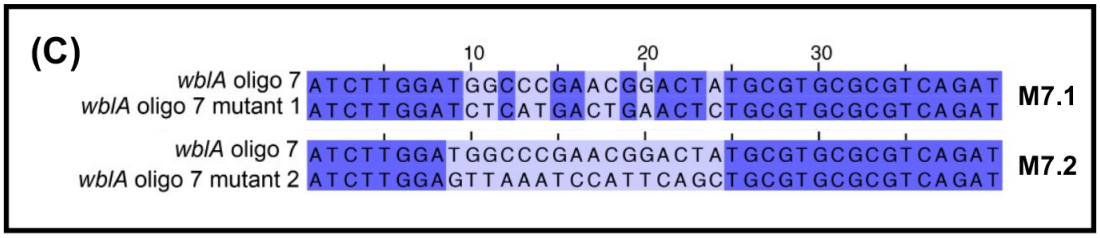
Table 1. OrrA binding sites in *S. venezuelae*. ChIP-seq against FLAG-tagged OrrA enriched these gene promoters (Figure S2) and the OrrA binding sites were identified for four of them *in vitro* using ReDCaT SPR. TMT proteomics data is shown for 18-hour cultures and indicate that OrrA activates expression of *wblA* and *vnz_04640* which encodes a conserved small hypothetical protein. N/D = not detected. *P-value not significant (>0.05).

Alignment of the OrrA binding sites identified using ReDCaT SPR enabled an OrrA consensus DNA recognition sequence to be generated using MEME (31) and this was validated by mutating conserved nucleotides in the ReDCaT SPR probes (Figures 3 and S4). The binding site appears to be palindromic which is consistent with dimeric OrrA binding to its target promoters.

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Figure 3. Identification of the OrrA binding site. (A). MEME generated consensus binding site for OrrA generated by aligning the four OrrA binding sites that were experimentally verified using ReDCaT SPR (Figure S4). (B). Binding of OrrA-DBD to the double stranded fragments of *wblA* promoter DNA was determined via ReDCaT SPR. Binding strength is represented as $\%R_{Max}$ values, calculated assuming two OrrA-DBDs binding to the imperfect palindromic MEME consensus; error bars shown (± SEM, n = 2). The key shows the concentrations of oligonucleotides used in SPR experiments. OrrA binds specifically to oligo probe 7 and mutation of the OrrA consensus site on probe 7 (M7.1 and M7.2) reduced OrrA binding to background levels. (C). Alignment of wild-type probe 7 with mutated probes M7.1 (partially mutated) and M7.2 (fully mutated). Note that the consensus is the reverse complement of the MEME consensus in A.

Tandem-mass-tag (TMT) proteomics analysis of the wild-type and Δ*orrA* strains grown for 18 hours on MYM agar show that the levels of WblA and Vnz_04640 are reduced in the Δ*orrA* mutant relative to wild-type (Figures 2 and S3 and Table 1; see Table S5 for the complete proteomics dataset). We conclude that OrrA directly activates the production of both proteins, which agrees with an earlier study that identified these genes as targets for *S. coelicolor* OrrA (13). Vnz_04640 is a small hypothetical 67 amino acid protein that is highly conserved in *Streptomyces* species and restricted to the phylum Actinomycetota (Figure S5). A previous study reported that over-expression of either *wblA* or the *vnz_04640* homologue *sco1375* in *S. coelicolor* only partially restores development in an *S. coelicolor* Δ*orrA* mutant (13). To test this in *S. venezuelae*, we over-expressed *vnz_04640* and *wblA* individually and together in the Δ*orrA* mutant. Over-expression of *vnz_04640* had no visible effect on the growth and development of either the wild-type or Δ*orrA* strains (Figure 4A). However, over-expression of either *wblA* or an artificial operon of *vnz_04640-wblA* fully restored sporulation to the Δ*orrA* mutant suggesting the Δ*orrA* developmental phenotype is due to the reduction in the levels of WblA (Figures 4B and C).

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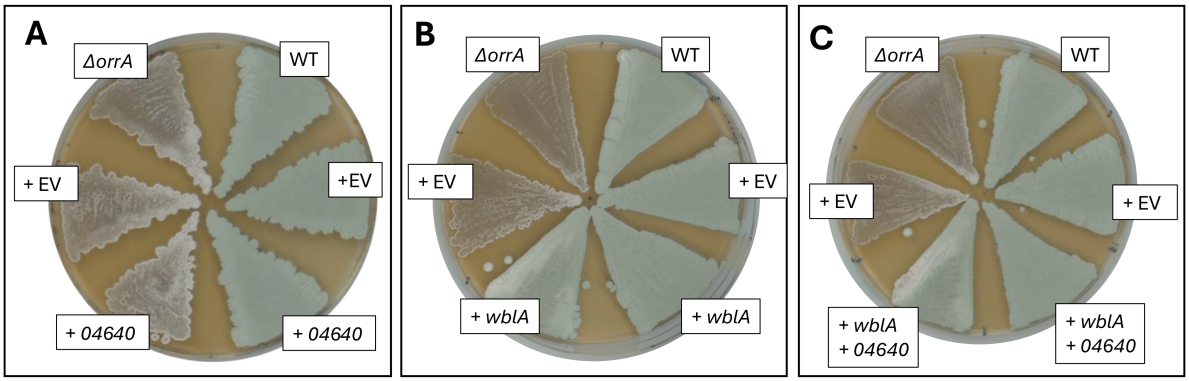


Figure 4. Over-expression of *wblA* restores normal sporulation to the Δ*orrA* mutant. (A). Wild-type and Δ*orrA* strains complemented in trans with either empty pIJ10257 (+EV) or pIJ10257 containing *vnz_04640* downstream of the *ermE** promoter. Cultures were grown for 3 days at 30 °C on MYM agar.

ChIP-seq and ReDCaT SPR data indicate that OrrA also binds upstream of the *vnz_16105-10* operon encoding putative drug resistance transporters and the *vnz_16115* gene which encodes a putative transcription factor that is a member of the σ^E regulon in *S. coelicolor* (Table 1) (32). Of these three genes, products were only detected in the TMT proteomics experiment for *vnz_16110* and *vnz_16115* and neither were significantly affected by the loss of OrrA (Table 1).

***S. venezuelae* Δ*orrA* and Δ*wblA* are defective in sporulation and over produce chloramphenicol.** The data in Figure 4 show that over-expression of *wblA* rescues the Δ*orrA* mutant and restores normal growth and sporulation suggesting a reduction in the levels of WblA results in the Δ*orrA* phenotype. To test this further in *S. venezuelae*, we deleted the *wblA* gene and observed that the mutant phenotype exhibits similar growth and development to the Δ*orrA* mutant in that it does not sporulate (Figure 5).

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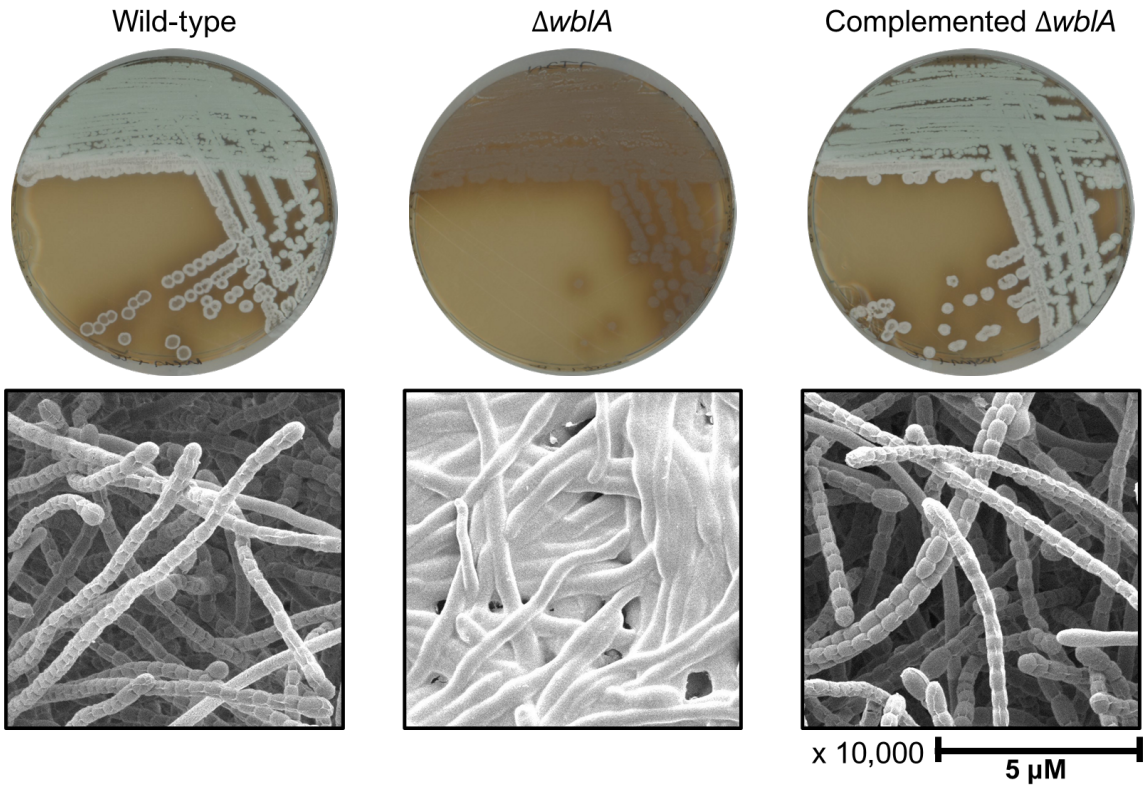


Figure 5. *S. venezuelae* Δ*wblA* does not sporulate. Top panels show MYM agar plate grown cultures of the wild-type (WT) and isogenic Δ*wblA* and Δ*wblA* complemented strains grown for three days. *S. venezuelae* spores are green, the WT and Δ*wblA* complemented strain are sporulating, whereas the Δ*wblA* mutant is not forming spores. Bottom panels show scanning electron micrographs of the same strains and confirm that the WT and Δ*wblA* complemented strain form aerial hyphae and spores, while the Δ*wblA* strain is only forming vegetative substrate mycelium.

Given that *S. coelicolor* Δ*orrA* and Δ*wblA* mutants both overproduce antibiotics (13), it was hypothesised that *S. venezuelae* Δ*orrA* would do the same. *S. venezuelae* makes the antibiotic chloramphenicol (33) and TMT proteomics data comparing the *S. venezuelae* wild-type and Δ*orrA* strains demonstrated that the entire chloramphenicol biosynthetic pathway is significantly upregulated in the *orrA* mutant (Figure 6A). We tested the antibacterial activities of *S. venezuelae* wild-type, Δ*orrA* and Δ*wblA* strains using agar plate bioassays. The results show that the wild-type strain does not inhibit *B. subtilis* while both mutants have clear zones of inhibition in overlay bioassays (Figure 6B). Next, we extracted cultures of the wild-type, Δ*orrA* and Δ*wblA* strains and showed by LCMS analysis that chloramphenicol is produced by both mutant strains but not by wild-type *S. venezuelae* (Figure 6C). These data indicate that

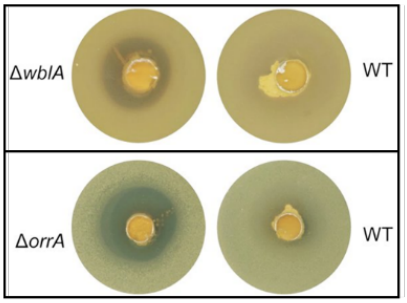
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the reduction in WblA levels in the Δ*orrA* strain is responsible for activating chloramphenicol biosynthesis.

(A)

Gene	Product	Function	Fold Change ΔorrA / WT
vnz_04400	CmlR	Pathway activator	4.317
vnz_04405	CmlL	Phosphopantetheinyl transferase	15.700
vnz_04410	CmlF	Ion antiporter	12.377
vnz_04415	CmlN	Cml efflux pump	4.444
vnz_04420	CmlE	DAHP synthase	15.832
vnz_04425	CmlD	4-amino-4-deoxychorismate mutase	8.323
vnz_04430	CmlC	4-amino-4-deoxychorismate dehydrogenase	9.411
vnz_04435	CmlB	4-amino-4-deoxychorismate synthase	17.539
vnz_04440	CmlA	Non-haem iron monooxygenase	10.213
vnz_04445	CmlP	Non-ribosomal peptide synthase	10.478
vnz_04450	CmlH	Amidase	9.442
vnz_04455	CmlI	N-oxidase	14.532
vnz_04460	CmlM	Acyl carrier protein	14.539
vnz_04465	CmlJ	Short chain dehydrogenase	9.669
vnz_04470	CmlK	Acyl-CoA-ACP synthetase, AMP-ligase	10.298
vnz_04475	CmlS	Flavin-dependent halogenase	9.694
vnz_04480	CmlT	Aldo-keto reductase	9.925

(B)



(C)

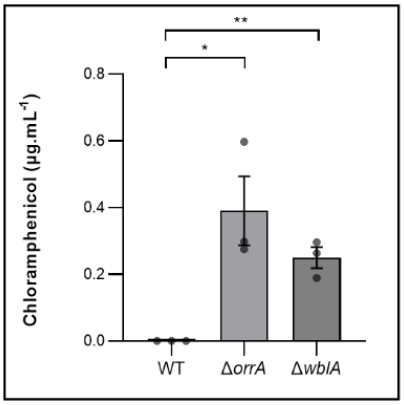


Figure 6. Chloramphenicol biosynthesis is upregulated in the Δ*orrA* mutant. (A). Significant fold changes in peptide abundance observed for the proteins encoded by the chloramphenicol BGC in the Δ*orrA* mutant relative to wild-type (WT) strains, quantified by TMT proteomics. (B). Agar plate bioassays showing colonies of wild-type S. venezuelae (WT) and the Δ*wblA* and Δ*orrA* mutants overlaid with soft agar containing the indicator strain *Bacillus subtilis*. The mutants inhibit *B. subtilis* but WT does not. (C). LCMS measurements of extracted cultures show that the Δ*orrA* and Δ*wblA* mutants are both producing the antibiotic chloramphenicol (n=3). *p*-values, * = 0.0198, ** p = 0.0140 (Unpaired *t*-test).

7. Concluding Remarks.

In this work we identified two genes under the direct control of the orphan response regulator OrrA in *S. venezuelae* NRRL B-65442, namely *wblA* and *vnz_04640.* We also showed that the Δ*orrA* and Δ*wblA* strains are phenotypically very similar, with an inability to sporulate and overproduction of the antibiotic chloramphenicol. Over-expression of *wblA* in the Δ*orrA* strain restored normal sporulation suggesting that the Δ*orrA* developmental phenotype is primarily due to a reduction in the cellular levels of WblA. We found that the Δ*wblA*

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phenotype is stable whereas the Δ*orrA* phenotype can revert to a strain that forms aerial hyphae (Figure S1).

A study of *S. coelicolor* also identified *wblA* and *sco1375* (the *vnz_04640* homologue) as OrrA target genes and we propose that OrrA function is highly conserved in the genus *Streptomyces*. Overexpression of *sco1375* or *wblA* partially restored normal development to *S. coelicolor* Δ*orrA* whereas overexpression of *vnz_04640* has no effect on the Δ*orrA* mutant and overexpression of *wblA* restored sporulation (Figure 4). WblA is required for normal aerial hyphae formation and sporulation (13,14) and it is a global regulator of antibiotic biosynthesis in *Streptomyces* species (19). Consistent with this, the deletion of *S. coelicolor orrA* leads to a reduction in WblA and an increase in actinorhodin and undecylprodigiosin biosynthesis in *S. coelicolor* while deletion of *S. venezuelae* Δ*orrA* reduces WblA levels and increases chloramphenicol biosynthesis (Figures 2 and 6).

The *sco1375* and *vnz_04640* genes encode small hypothetical proteins that contain 67 and 68 amino acids, respectively, and both contain DUF5999, a domain of unknown function which is predominantly found in the phylum Actinomycetota. The function of this protein is not known but it is highly conserved in *Streptomyces* species, as are OrrA and WblA, suggesting they have conserved functions (Figure S5), with OrrA solely dedicated to activating the production of Vnz_04640 and WblA (Figures 2 and S3). Future work will be aimed at investigating the functions of Vnz_04640 and WblA in *S. venezuelae.*

8. Author statements

8.1 Author contributions

Conceptualisation: KRT, LB, NAH, BW and MIH

Data curation: KRT, LB, NAH, ADMB, CA, GC, GS, RS, BW and MIH

Formal analysis: KRT, LB, NAH, ADMB, GC, GS

Funding acquisition: BW and MIH

Investigation: KRT, NAH, ADMB, CA

Methodology: KRT, NAH, LB, CA RS, GC, GS

Project administration: BW, MIH

Resources: RS, GS, BW, MIH

Software: GC, GS

Supervision: LB, BW and MIH

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Visualisation: KRT

Writing – original draft: KRT and MIH

Writing – review and editing: all authors.

8.2 Conflicts of interest

The authors declare that there are no conflicts of interest.

8.3 Funding information

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8.4 Ethical approval

N/A

8.5 Consent for publication

N/A

8.6 Acknowledgements

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