MtrA is an essential regulator that coordinates antibiotic production and development in *Streptomyces* species

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Abstract. *Streptomyces* bacteria make numerous secondary metabolites, including half of all known antibiotics. Production is coordinated with their complex life cycles but the regulators that coordinate development with antibiotic biosynthesis are largely unknown. This is important to understand because most *Streptomyces* secondary metabolites are not produced under laboratory conditions and unlocking the 'cryptic' biosynthetic gene clusters (BGCs) is a major focus for antibiotic discovery. Here we characterise the highly conserved actinobacterial response regulator MtrA in *Streptomyces* species. MtrA is essential and regulates cell cycle progression in *Mycobacterium tuberculosis*. We show that MtrA is also essential in *Streptomyces venezuelae* where it controls genes required for DNA replication and cell division. MtrA also directly regulates the expression of genes in >70% of its BGCs and artificially activating MtrA switches on the production of antibiotics in *S. coelicolor and S. venezuelae*. We propose that MtrA coordinates antibiotic production and development in *Streptomyces* species.

Introduction.

Streptomyces secondary metabolites account for two thirds of all known antibiotics and numerous other compounds that are used in human medicine as anticancer, anti-parasitic, antiviral and immunosuppressant drugs. Discovery of these natural products peaked in the 1950s but there has been a resurgence of interest in the 21st century, driven by genome sequencing and the increasing threat of drug resistant infections (Katz & Baltz 2016). Despite their importance however, we still have a poor understanding of how *Streptomyces* bacteria control the production of their secondary metabolites. This is important because ≥75% of their secondary metabolite biosynthetic gene clusters (BGCs) are not expressed in laboratory culture and activating cryptic BGCs could facilitate the discovery of new antibiotics and other useful compounds (Hosaka et al. 2009; Rutledge & Challis 2015).

The major way in which bacteria sense and respond to their environment is through two-component systems and several have been implicated in the regulation of antibiotic production in *Streptomyces* species (Hsiao & Kirby 2009). Two component systems typically

consist of a bifunctional sensor kinase and a cognate response regulator (Salazar & Laub 2015). The sensor kinase perceives an extracellular signal and activates its cognate response regulator through a two-step phosphorylation. The phosphorylated regulator (RR~P) brings about a response to the original signal, usually by modulating target gene expression. In the absence of signal, the bifunctional sensor kinase dephosphorylates its response regulator to keep the response switched off (Capra & Laub 2012). The delicate balance of kinase and phosphatase activities is crucial in modulating the activity of the response regulator and its target genes during the bacterial cell cycle (Salazar & Laub 2015). Cross-talk between two component systems is rare in wild-type cells but removal of a sensor kinase can result in constitutive activation of its response regulator by a non-cognate sensor kinase or by the cellular pool of acetyl phosphate (Hutchings, Hong & Buttner 2006). Similarly, altering a sensor kinase to block its phosphatase activity can result in a response regulator that cannot be dephosphorylated and is rendered constitutively active (Salazar & Laub 2015).

Here we report characterisation of the highly conserved actinobacterial response regulator MtrA in the model organism *Streptomyces venezuelae* (Hoskisson & Hutchings 2006). MtrA was first identified as an essential regulator in *Mycobacterium tuberculosis* (Mycobacterium tuberculosis regulator A) (Zahrt & Deretic 2000). *M. tuberculosis* MtrA (TB-MtrA) regulates the expression of *dnaA* and *dnaN*, which are essential for DNA replication, and sequesters the origin of DNA replication, *oriC*, in dividing cells (Purushotham et al. 2015). TB-MtrA also regulates the expression of cell division genes and interacts directly with the DnaA protein, which presumably prevents the initiation of DNA replication (Purushotham et al. 2015). The MtrB sensor kinase is activated following localisation to the site of cell division through interaction with FtsI and DivIVA (Wag31) and these data have led to a model in which oscillations in TB-MtrA~P levels play a key role in cell cycle progression by repressing DNA replication and activating cell division (Purushotham et al. 2015; Plocinska et al. 2012; Plocinska et al. 2014). An accessory lipoprotein called LpqB modulates the activity of MtrB in mycobacteria and an *M. smegmatis* Δ*lpqB* mutant grows as *Streptomyces*-like filaments (Nguyen et al. 2010). In the closely related *Corynebacterium glutamicum*, Cg-MtrA controls

genes involved in cell wall remodelling and the osmotic stress response (Brocker, Mack & Bott 2011a; Krämer 2009) but deletion of the *mtrAB* genes gives rise to elongated cells which are indicative of a defect in cell division (Möker et al. 2004).

Streptomyces bacteria are filamentous saprophytes which grow through the soil as branching substrate mycelium that extends at the hyphal tips. Nutrient starvation triggers the production of reproductive aerial hyphae that accelerate DNA replication, generating up to 200 copies of the chromosome in each aerial hypha, before undergoing cell division to form chains of unigenomic spores. Aerial hyphae production and sporulation are coordinated with the production of bioactive secondary metabolites, including antibiotics. *S. coelicolor* has traditionally been used to study development and antibiotic production because it makes pigmented antibiotics and spores but it only differentiates into aerial hyphae and spores when grown on solid agar. *S. venezuelae* has recently emerged as an alternative model for studying development because it completes a full developmental life cycle in liquid growth medium in ~20 hours (Bush et al. 2015). Here we present evidence that MtrA is essential for the growth of *S. venezuelae* and that it directly regulates the expression of genes involved in DNA replication, cell division and secondary metabolism, several of which (*dnaA*, *dnaN*, *oriC* and *wblE/whiB1*) are also regulated by TB-MtrA. We performed ChIP-seq throughout the *S. venezuelae* life-cycle and found that the DNA binding activity of Sv-MtrA displays a biphasic plasticity such that it is active during vegetative growth and sporulation, but inactive during formation of aerial hyphae. Sv-MtrA also binds to sites spanning ~85% of the BGCs in *S. venezuelae* and directly modulates expression of target genes in at least 75% of these BGCs. ChIP-seq in *S. coelicolor* revealed that Sc-MtrA binds to many of the same cell division targets and to genes spanning ~70% of its BGCs. Key developmental genes bound by MtrA in both streptomycetes include *bldM, ftsZ, ssgA, smc, smeA, whiI, whiB, whiD* and *wblE.* Deletion of *mtrB* constitutively activates MtrA and its target genes and leads to constitutive production of antibiotics in *S. coelicolor* and *S. venezuelae.* Finally, we show that introducing TB-MtrA and gain of function TB-MtrA and Sv-MtrA proteins into *S. venezuelae* also activates antibiotic

production. We propose that manipulating the activity of MtrA could be a route to the activation of cryptic BGCs in all *Streptomyces* species.

Results

MtrA is essential in *Streptomyces venezuelae*.

To investigate the function of the MtrAB two component system in *S. venezuelae* we deleted either *mtrA* or *mtrB* (Gust et al. 2003). *mtrB* was deleted easily but all attempts to delete *mtrA* were unsuccessful until we introduced a second copy of *mtrA in trans.* The same result was reported for TB-MtrA and the authors concluded that it is essential in *M. tuberculosis* (Zahrt & Deretic 2000). It follows that if MtrA is essential in these bacteria then it must be active in the absence of MtrB, otherwise deleting *mtrB* would also be lethal. We propose that deletion of *mtrB* leads to constitutive phosphorylation of MtrA, by the cellular pool of acetyl phosphate or another sensor kinase. MtrA~P has been shown to be the active form for DNA binding in both *C. glutamicum* and *M. tuberculosis* (Fol et al. 2006; Brocker, Mack & Bott 2011b). We observed a similar result with *Streptomyces coelicolor* VanRS, where VanR and its target genes are constitutively active in a Δ*vanS* mutant (Hutchings, Hong & Buttner 2006). Surprisingly, deletion of *mtrB* has no effect on growth rate or development in liquid cultures but in agar grown cultures, deletion of Δ*mtrB* results in a sporulation defect in the centre of the colonies (Figures S1 and S2).

MtrA activity changes during the life cycle of *S. venezuelae*.

Published microarray data for *S. venezuelae* NRRL B-65442 (Bibb et al. 2012) (ArrayExpress accession E-MEXP-3612), shows that *mtrA* expression levels are highest during vegetative growth at 10 and 12 hours and drop approximately 2-fold at 14-20 hours growth (Figure S3). RNA sequencing at 12, 16 and 20 hours (accession number = GSE81104), shows a dip in transcript levels at 16 hours (aerial growth) and an increase following sporulation, at 20 hours (Figure S4). Thus, the *mtrA* gene appears to be most highly transcribed during active growth and following sporulation. We did not detect any significant change in MtrA protein levels

during the life cycle using immunoblotting (Figure S3) and attempts to compare levels of phosphorylated and non-phosphorylated MtrA using Phostag were unsuccessful (not shown). To examine MtrA activity during the *S. venezuelae* life cycle, we performed ChIP-seq throughout development. We introduced an expression construct for MtrA-3xFlag *in trans* under the control of its own promoter and then deleted the native *mtrA* gene. Survival of this strain shows that MtrA-3xFlag is functional and there are no significant difference in growth rates between this strain and the wild-type (Figures S5 and S6). We performed ChIP-seq on the wild-type (control) and the MtrA-3xFlag strain at 8, 10, 12, 14, 16, 18 and 20 hours to examine the DNA binding activity of MtrA at key stages of the developmental life cycle (accession number GSE84311, Table S1). The 8-12h time points correspond to the active growth of substrate hyphae, between 14-18 hours they produce aerial hyphae and undergo DNA replication and cell division to form spores and at 20 hours the culture is made up of immature and mature spore chains. We mapped the reads to the *S. venezuelae* NRRL B-65442 genome sequence (Genbank accession CP018074). Analysis of the ChIP-seq data using a *P* value > 0.05 revealed that only one target is enriched at all time points, the promoter region of the *ectABCD* operon, which is the BGC for the secondary metabolites ectoine and 5' hydroxyectoine (5HE). We could not detect ectoine or 5HE in the wild-type or Δ*mtrB* mutant using LCMS with authentic standards so we assume that MtrA~P represses *ectABCD* expression throughout the life cycle. Analysis of all the ChIP-seq datasets using *P* > 0.05 shows that the pattern of binding of MtrA is dynamic throughout the lifecycle with peak binding activity seen at 10 and 20 hours, coinciding with mid-exponential growth and within spores (Figure S7). This is more consistent with *mtrA* expressison levels observed in the dRNA-seq experiment than in the microarray data (Figures S3-4). This may be because the microarray experiments were performed in a different laboratory so growth rates may vary whereas we performed the RNA-seq experiments under identical conditions. However, these data show that the DNA binding activity of MtrA changes during the life cycle and mapping ChIP-seq reads at targets such as the *mtrA* promoter provides a good illustration of this biphasic activity (Figure 1). Differential RNA sequencing also shows that the *mtrAB-lpqB* genes are transcribed

as a single leaderless transcript and that MtrA binds immediately upstream of the -35 region, suggesting positive autoregulation. There is a second putative transcript start site at -79bp, which is upstream of the MtrA binding site, suggesting there may be two promoters driving expression of *mtrAB-lpqB* (Figure 1).

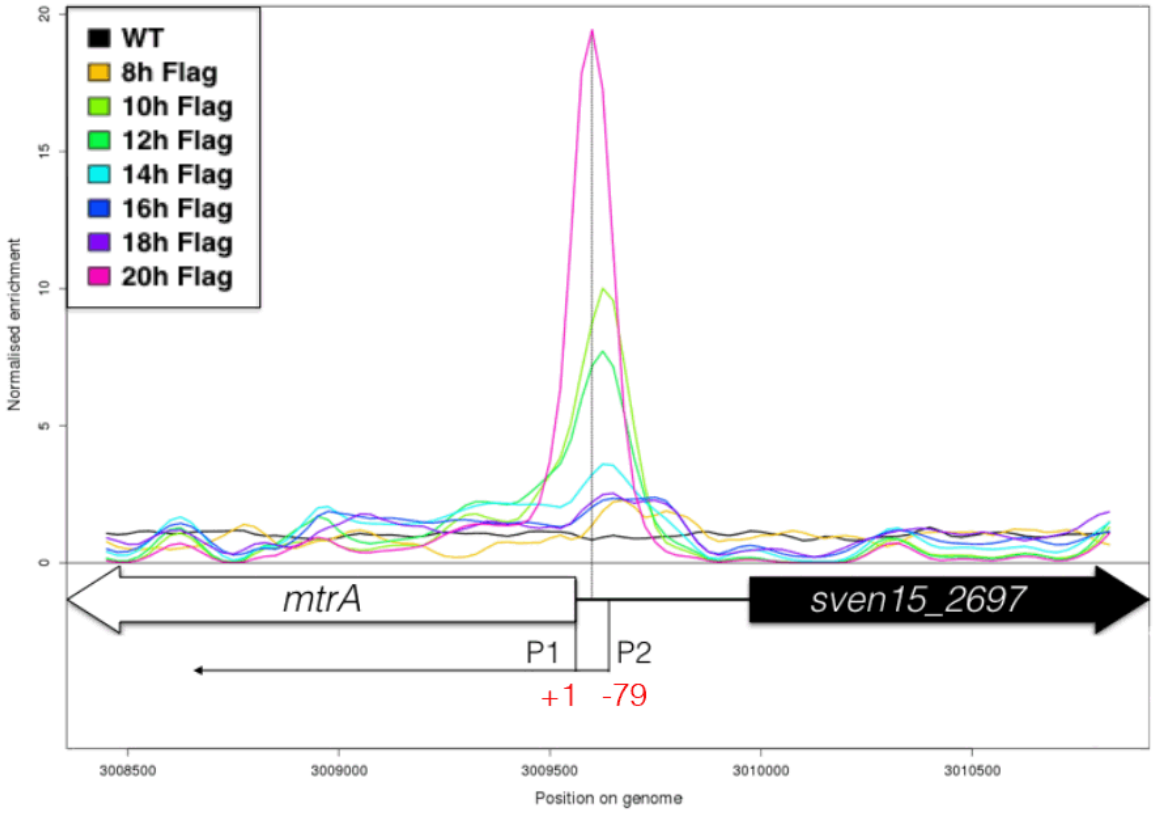

Figure 1. MtrA autoregulates its own expression. *ChIP-seq during the Streptomyces venezuelae life cycle shows that MtrA has highest activity in actively growing mycelium (10 and 12 hours) and in spores (20 hours). The peaks shown are at the mtrAB-lpqB operon promoter region which has two transcript start sites at +1 (P1) and -79 (P2). The position of the MtrA ChIP peak suggests it activates P1 and blocks P2.*

Identifying the MtrA binding site. To identify an MtrA consensus binding sequence we performed MEME analysis (Bailey et al. 2015) on three MtrA target promoters identified by ChIP-seq and bound by purified MtrA using *in vitro* electrophoretic mobility shift assays (EMSAs). These analyses suggested that MtrA recognises and binds to an AT-rich 7 bp motif. We then took 50 bp of sequence from beneath each peak at the 14h and the 16/18h time points and ran them through MEME to identify conserved motifs. This analysis suggests that

MtrA binds a direct repeat of the AT rich motif separated by a 5bp spacer (Figure 2). However, many of the sequences enriched in the MtrA ChIP-seq dataset do not contain this motif and we hypothesise that MtrA binds target sites directly and also by interacting with other transcription factors. Immunoprecipitation experiments using anti-Flag beads pulled down four different regulators in the MtrA-3xFlag cultures that are not immunoprecipitated in the wild-type control. The genes encoding these regulators (*sven15_0243*, *sven15_2691*, *sven15_3571* and *sven15_4644*) are also targets for MtrA (Table S1). Sven15_3571 is DnaA, which initiates DNA replication and acts as a transcription factor in bacteria. TB-MtrA also interacts with DnaA although the effects of this interaction are not known (Purushotham et al. 2015). Thus, our data suggests (but does not prove) that MtrA forms complexes with other DNA binding proteins and this may explain why there are so many enrichment peaks in the 20-hour ChIP-seq dataset. Interaction between regulatory proteins is not unprecedented in *S. venezuelae.* The response regulator BldM modulates one set of target genes as a homodimer and another set by forming heterodimers with the WhiI response regulator (Bassam et al. 2014). The developmental regulators WhiA and WhiB also proposed to interact and have identical ChIP-seq regulons (Bush et al. 2016).

MtrA regulates the expression of DNA replication and cell division genes.

ChIP-seq shows that MtrA activity changes during the *S. venezuelae* life cycle (Figure 1). RNA sequencing of wild-type and Δ*mtrB* strains shows that expression of *mtrA* is 3-fold upregulated in the Δ*mtrB* mutant (Table S2) which is consistent with activation by MtrA~P. Given the essentiality of MtrA and its altered activities at different stages of the life cycle we predicted that it could play a key role in regulating cell cycle progression in *S. venezuelae*, as in *M. tuberculosis* (Purushotham et al. 2015; Plocinska et al. 2014). Indeed, ChIP-seq shows that several key developmental genes are bound by MtrA in *S. venezuelae* and *S. coelicolor* (see Tables S1 and S2 for complete lists).

Target	P value	Binding site
Sven15_0205 (ectA)	4.23e-5	GCGTTACCCATCCGTTATAAACGGTCAT
Sven15_0880 (*cmlF*)	4.23e-5	ATTCGCCATCTACGTTCTCGGGCTGGCC
Sven15_3571 (*dnaA*)	1.51e-5	CACAATCTTTTCCGTTCTGTCCTTACCT

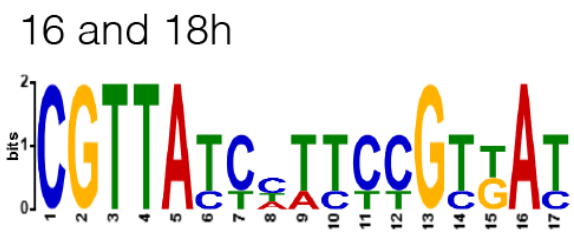

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Figure 2. MtrA binds to 6-5-6bp direct repeat motif. *The table shows three MtrA ChIP targets that are bound in vitro by purified MtrA protein. They are the promoter regions of ectABCD operon (ectoine BGC), cmlF (chloramphenicol transporter) and dnaA (transcription factor and initiator of DNA replication). MEME analysis identified a conserved 7bp motif (coloured) that is present as a 6-5-6bp repeat motif at the ectA promoter (second half underlined). MEME analysis of different subsets of ChIP targets identified the same direct repeat motif (shown for the targets identified at 14 hours and 16 and 18 hours.*

205 Targets common to both TB- and Sv-MtrA are the promoter regions of *wblE*, *dnaA* and *dnaN*,
206 and the *oriC* region between *dnaA* and *dnaN* (Figure 3A). RNA-seq shows that deletion of
207 *mtrB* does not affect the expression levels of *dnaA* or *dnaN* under the conditions used but
208 deletion of *mtrB* causes a two-fold increase in *wblE* transcript levels, suggesting MtrA-
209 dependent activation. MtrA also directly represses *adpA* expression, which is down-regulated
210 nearly 4-fold in the Δ*mtrB* mutant (Table S2). AdpA controls key developmental genes and
211 represses DNA replication in *Streptomyces* species (Wolański et al. 2012; Wolański et al.
212 2011). *wblE* encodes a WhiB-like transcription factor of unknown function that is homologous
213 to WhiB1 in *M. tuberculosis.* Both *wblE* and *whiB1* are essential (Stapleton et al. 2012). Sv-
214 MtrA also binds upstream of the cell division genes *ssgA* and *ssgB*, which are upregulated in
215 the Δ*mtrB* mutant, and *ftsZ* which is unaffected by loss of MtrB, probably because this gene
216 is subject to complex regulation (Figure 3B and Table S2).

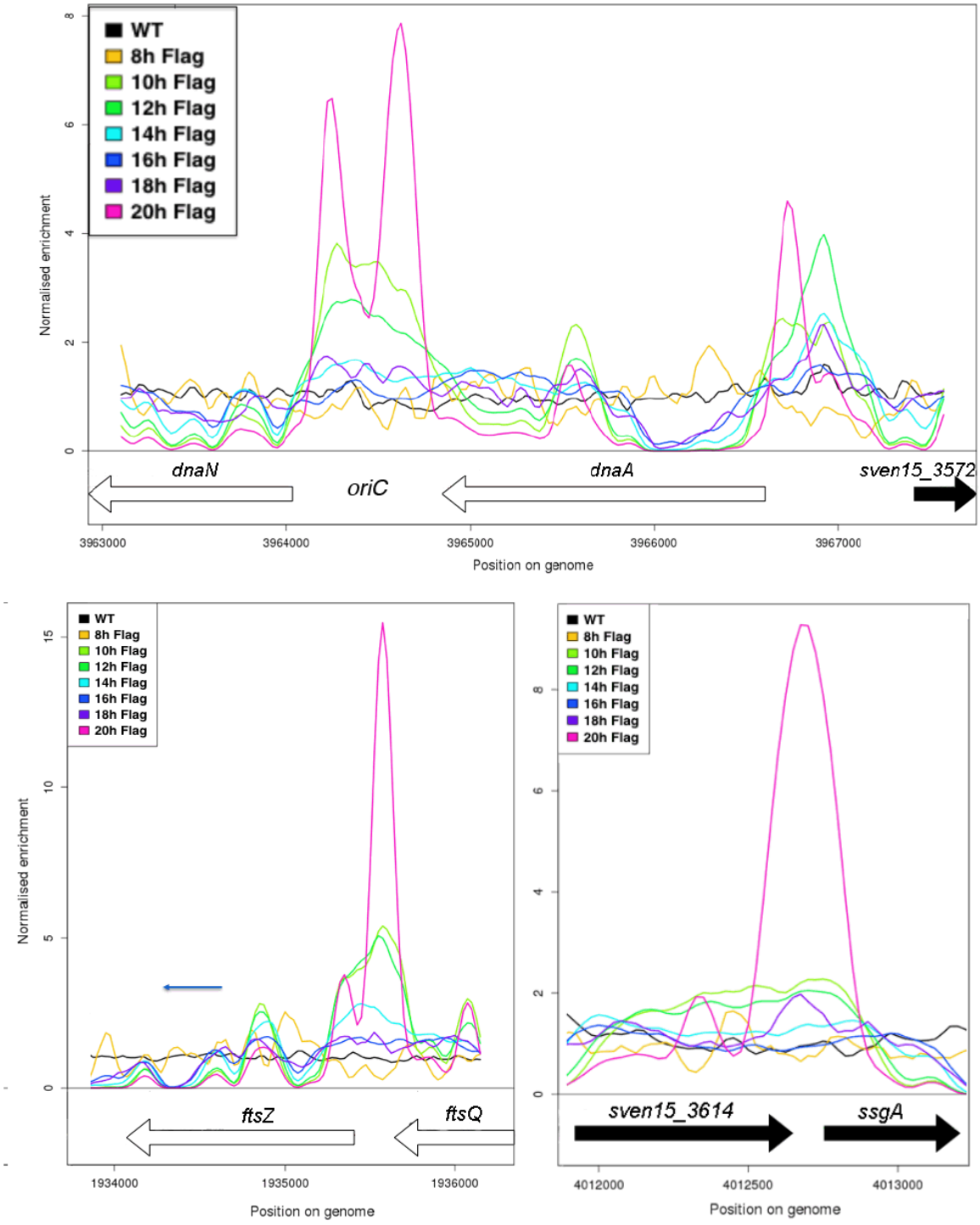

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Figure 3. MtrA binds to targets required for DNA replication and cell division in S. venezuelae. *Top. MtrA ChIP peaks upstream of dnaA, which encodes the initiator of DNA replication, and dnaN which encodes the DNA polymerase beta clamp subunit and at the origin of DNA replication, oriC. Bottom. MtrA ChIP peaks upstream of ftsZ, which encodes the Z ring forming FtsZ protein and ssgA, whose product helps localise FtsZ to the sites of cell division during sporulation.*

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SsgAB localise the divisome marker protein FtsZ to the correct positions in aerial hyphae to mark the sites of cell division, prior to sporulation (Schwedock et al. 1997; Willemse et al. 2011). Sv-MtrA also regulates expression of the *smeA-ssfA* operon, which is elevated between 2- and 4-fold in the Δ*mtrB* mutant. SmeA targets the DNA pump SffA to the cell division septa (Bush et al. 2015). MtrA also binds upstream of (and activates some) key developmental regulators, including *whiB*, *whiD*, *whiI* and *bldM* (Table S2) (Bassam et al. 2014; Molle et al. 2000; Bush et al. 2016). Many of these Sv-MtrA target genes are also repressed by BldD (in complex with c-di-GMP), a master regulator of *Streptomyces* development. Sv-MtrA does not regulate *bldD* expression but it does regulate the expression of genes encoding proteins that metabolise cyclic di-GMP, which is required for BldD-mediated repression (Tschowri et al. 2014) (Table S2).

MtrA regulates global BGC expression in *Streptomyces venezuelae*.

ChIP-seq shows that MtrA binds to sites spanning 28 of the 31 BGCs predicted by antiSMASH 3.0 in the *S. venezuelae* genome (Weber et al. 2015) (Table S3). The only BGCs with no MtrA enrichment are those encoding biosynthesis of the desferrioxamine siderophores, the WhiE polyketide spore pigment and a putative insecticidal complex. Of these three clusters, the WhiE BGC is upregulated in the Δ*mtrB* mutant suggesting indirect regulation by MtrA, probably via BldM (Bassam et al. 2014). The other two BGCs are unaffected by loss of MtrB (Table S3). Of the 28 BGCs that are bound by MtrA, nine have genes that are positively regulated by MtrA, 10 have genes that are negatively regulated by MtrA and three are subject to both positive and negative regulation by MtrA at individual genes within the gene cluster (Table S3). The other six BGCs have sites that are enriched in MtrA ChIP-seq but expression of the genes nearest the ChIP-seq peaks are not affected by deletion of *mtrB* under the conditions we used (Table S3). We predict the *ectABCD* operon is repressed by MtrA because the *ectA* promoter is bound by MtrA at all time points. Consistent with this, we cannot detect ectoine or 5HE in the wild-type or Δ*mtrB* strains. Since most of the *S. venezuelae* BGCs are

uncharacterised we know little about their cluster specific regulation, or their natural products. It should also be noted that the antiSMASH predictions we have used here likely include additional flanking genes that may not be part of the BGC so the actual number of BGCs bound by MtrA may be slightly lower.

MtrA activates chloramphenicol production in *S. venezuelae.*

Phenotypic screening of the Δ*mtrB* mutant revealed that a cryptic antibacterial is activated by removing MtrB whereas over-expressing *mtrA* in wild-type *S. venezuelae* has no effect (Figure S8). This is probably because MtrA~P levels only increase in the absence of MtrB. *S. venezuelae* encodes the biosynthetic pathway for the antibiotic chloramphenicol, which according to previous studies is off in the wild-type strain (Fernández-Martínez et al. 2014). ChIP-seq shows that Sv-MtrA binds in the intergenic region between the divergent *cmlF* (*sven15_0879*) and *cmlN* (*sven15_0880*) genes (Figure 4) and expression of *cmlN* is 6-fold upregulated in the Δ*mtrB* mutant, consistent with direct activation by MtrA. CmlN is an efflux permease, predicted to export chloramphenicol (Fernández-Martínez et al. 2014). HPLC confirmed that chloramphenicol is produced in the Δ*mtrB* mutant but we also detected very low levels in the wild-type strain suggesting the cluster is not completely silent (Figure 4). Cultivation for 24 hours in biological and technical triplicates confirmed an increased production of chloramphenicol in the Δ*mtrB* mutant with a mean concentration of 0.41 mg/L which is >30 times higher than the wild-type strain (0.013 mg/L) or wild-type over-expressing *mtrA* (0.010 mg/L).

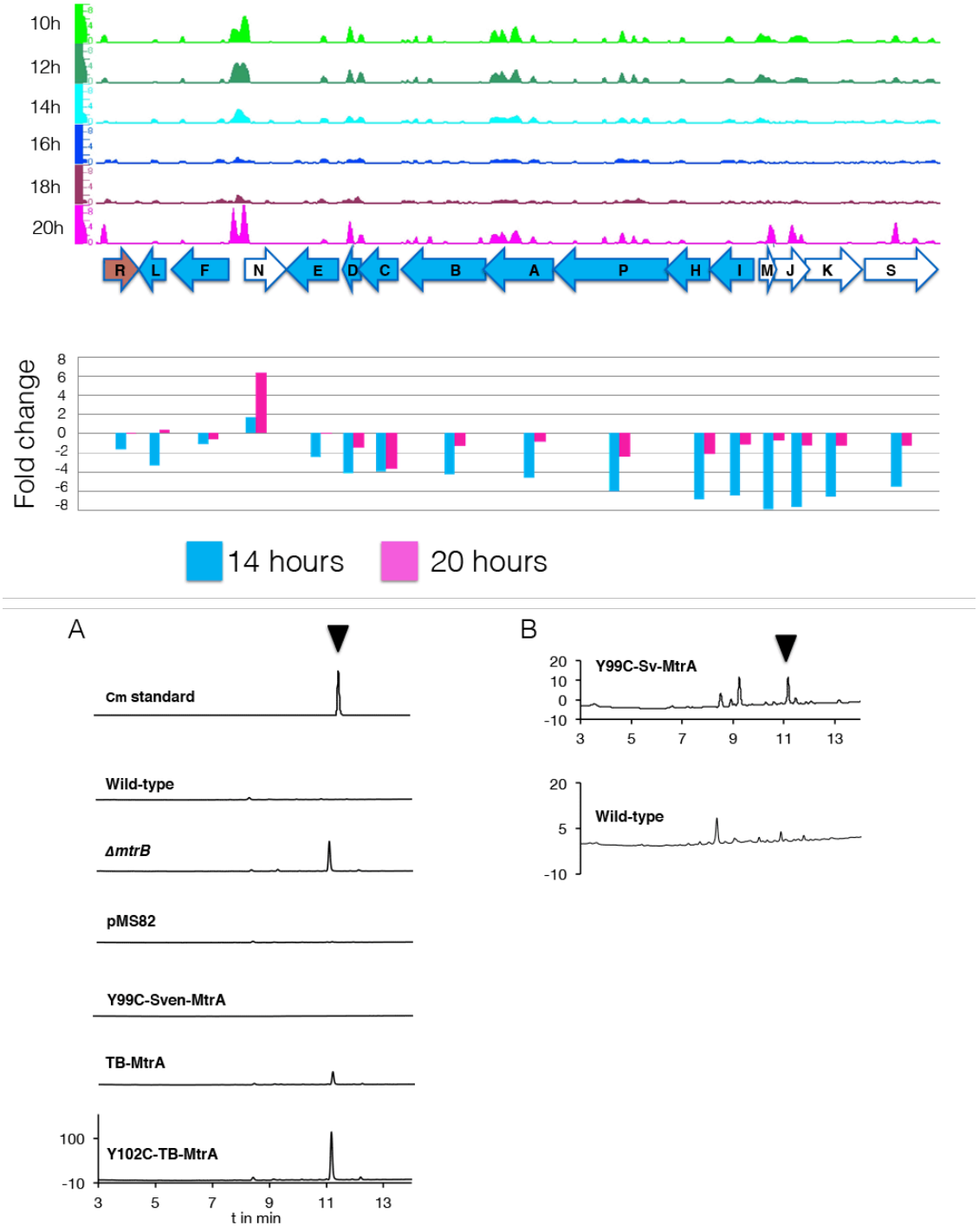

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Figure 4. MtrA controls chloramphenicol production. *Top. MtrA ChIP peaks at the chloramphenicol (Cm) BGC show two significant peaks between cmlN and cmlF and a smaller peak upstream of the regulator cmlR which is P<0.05. Middle. Expression data for wild-type and ΔmtrB strains at 14 and 20 hours shows cmlF is 6-fold upregulated in ΔmtrB suggesting it is activated by MtrA. All the other Cm genes are downregulated in the mutant which may be due to negative feedback. Bottom left. HPLC shows the Cm standard and extracts of wild-type, ΔmtrB, wild-type plus empty pMS82 vector, and wild-type plus pMS82 expressing Sv-Y99C MtrA, TB-MtrA and TB-Y102C MtrA. Right. Zooming in shows that Sv-Y99C MtrA induces production of low levels of Cm production.*

268 **Deleting *mtrB* has a global effect on the *S. venezuelae* metabolome.**

269 While loss of MtrB clearly leads to increased production of chloramphenicol, we were
270 interested to know if there are any other effects on the metabolome. We cultivated the wild
271 type strain and three independently isolated *ΔmtrB* mutants in biological and technical
272 triplicates and analysed the extracts by UPLC/HRMS using an untargeted metabolomics
273 approach. Runs were aligned to compensate for between-run variation and a peak-picking
274 algorithm was applied to allow for the immaculate matching of each feature (a discrete *m*/*z*
275 value and its retention time) among all runs. Following normalisation, features could be
276 compared quantitatively and their putative identity proposed based on their high-resolution
277 MS-signal. Comparing the level of metabolite signals, it appeared that all *ΔmtrB* mutants
278 showed increased production of a considerable portion of their putative secondary
279 metabolites. To display multidimensional data, we used Principle Component Analysis (PCA)
280 (Figure 5). Each sphere in the 3D Plot represents one dataset obtained from a particular
281 UPLC-HRMS run. Data from the *ΔmtrB* mutant strains clearly group together, and are distinct
282 from data obtained from the wild type, while variations within each group are comparably
283 small. The 3D Plot therefore shows consistent and global changes in the metabolome upon
284 loss of MtrB (Figure 5).
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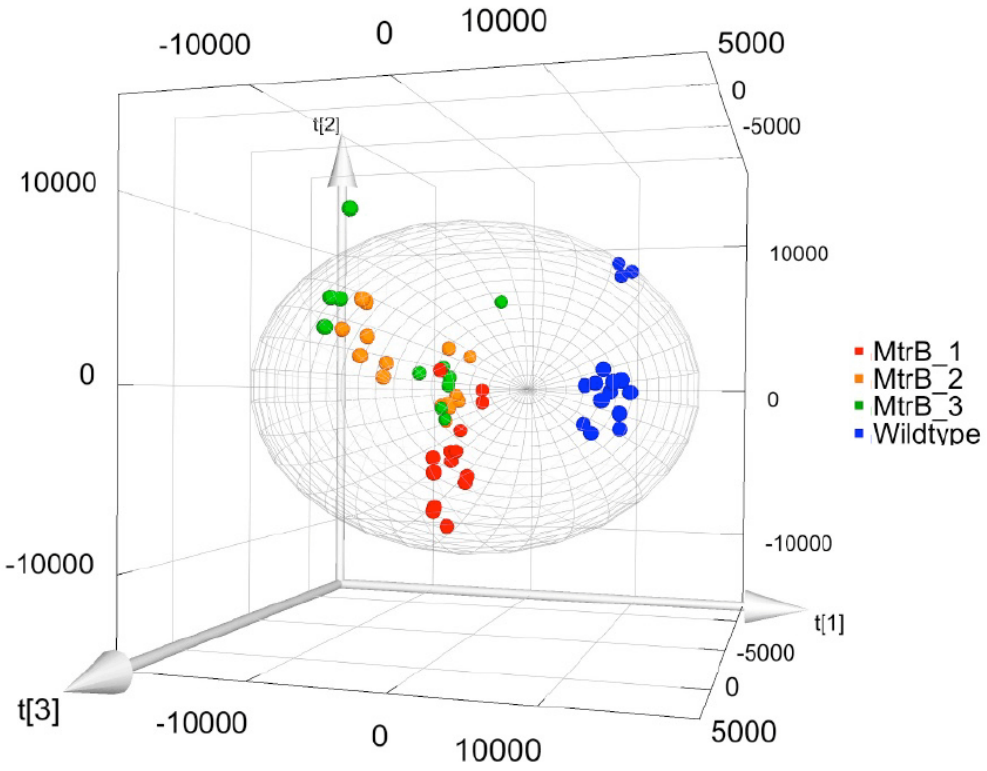

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Figure 5. Loss of MtrB results in a global shift in the S. venezuelae metabolome. *Principle Component Analysis on the wild-type (blue dots) and triplicate samples of the ΔmtrB strain (red, green and orange dots). Data from ΔmtrB mutant strains clearly group together, and are distinct from data obtained from the wildtype while variations within each group are comparably small.*

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288 **MtrA directly activates antibiotic production in *Streptomyces coelicolor.***

289 MtrA is conserved in all sequenced *Streptomyces* genomes so we reasoned that it might
290 activate antibiotic production in other streptomycetes. To test this, we deleted *mtrB* in the
291 model organism *Streptomyces coelicolor* which is probably the best characterised species in
292 terms of its secondary metabolite BGCs (Challis 2013). The 16S rDNA phylogenetic tree of
293 the family *Streptomycetaceae* shows that *S. venezuelae* (clade 40) is highly divergent from *S.*
294 *coelicolor* (clade 112) so we reasoned that if deleting *mtrB* switches on antibiotic production
295 in these distantly related species it may be universal to all streptomycetes (Labeda et al. 2011).
296 The *S. coelicolor ΔmtrB* mutant forms small colonies that are delayed in sporulation but normal
297 growth and differentiation are restored by reintroducing *mtrB in trans* (Figures S9-10). The
298 Δ*mtrB* mutant over-produces the red antibiotic undecylprodigiosin and the blue antibiotic

299 actinorhodin and we also detected significant amounts of metacycloprodigiosin, a potent
300 anticancer compound encoded by the undecylprodigiosin BGC but not previously reported
301 from *S. coelicolor* (Figure 6). Other secondary metabolites are clearly down-regulated in the
302 Δ*mtrB* mutant, including the desferrioxamine B and E siderophores and germicidin A. We
303 performed ChIP-seq in *S. coelicolor* because its BGCs and their natural products are the most
304 well defined of any *Streptomyces* species (Bentley et al. 2002; Challis 2013; van Keulen &
305 Dyson 2014). The results show that MtrA binds to sites spanning 21 of its 29 BGCs (Tables
306 S4 and S5). MtrA binds upstream of the genes encoding the actinorhodin activator ActII-4 and
307 the undecylprodigiosin activator RedZ (Figure S11). MtrA does not bind to the desferrioxamine
308 BGC (*desABCD*) in *S. coelicolor* (Table S4) but does bind upstream of *sco4394*, which
309 encodes DesR, an iron dependent repressor of desferrioxamine biosynthesis (Figure S11). A
310 single type III polyketide gene, *SCO7221*, is responsible for germicidin biosynthesis (Song et
311 al. 2006) and it is not regulated by MtrA, so the effect on germicidin biosynthesis must be
312 indirect.
313
314 ***M. tuberculosis* MtrA activates chloramphenicol production in *S. venezuelae*.**
315 Modulating MtrA activity by deleting *mtrB* to switch on antibiotic production is time consuming
316 and impractical, particularly when applied to multiple *Streptomyces* strains. To test whether
317 gain-of-function MtrA proteins might be used instead we constructed *Streptomyces* expression
318 vectors for wild-type TB-MtrA, gain-of-function Y102C TB-MtrA (Satsangi et al. 2013) and a
319 Y99C Sv-MtrA variant which has the equivalent change to Y102C TB-MtrA (Figure S12). We
320 were unable to delete the native *mtrA* gene in strains carrying these constructs but all three
321 induced chloramphenicol production in the wild-type strain (Figure 4). Y99C Sv-MtrA switches
322 on the production of relatively small amounts of chloramphenicol in wild-type *S. venezuelae*
323 but the TB-MtrA proteins switch on much higher levels of production, with the strain expressing
324 Y102C-TB-MtrA making more chloramphenicol than the Δ*mtrB* mutant (Figure 4). These
325 results suggest that expression of different MtrA proteins is a useful avenue to explore in terms
326 of activating BGCs in *Streptomyces* species and could be useful tools for the community. The

MtrA expression constructs described in this work are available from AddGene (IDs 85988-94).

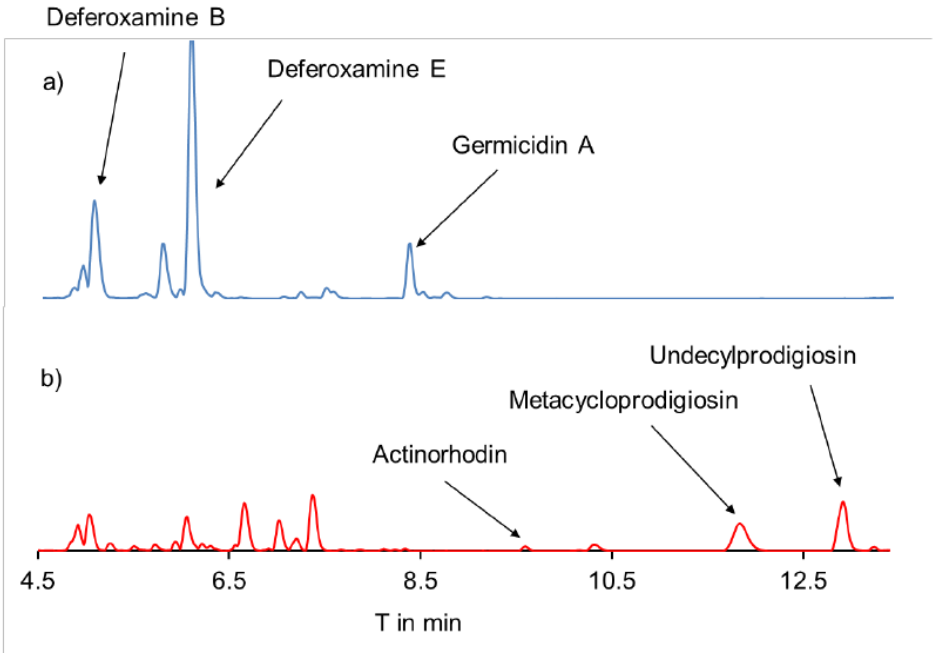

Figure 6. Deleting mtrB switches on antibiotic production in S. coelicolor. *(a) HPLC on extracts of wild-type S. coelicolor M145 cultures shows production of desferrioxamines and germicidin. (b) HPLC on extracts of an S. coelicolor ΔmtrB mutant shows production of actinorhodin and undecylprodigiosin is switched on while the production of the desferrioxamine siderophores and germicidin are switched off.*

Discussion.

This work describes the first detailed analysis of the highly conserved actinobacterial response regulator MtrA in *Streptomyces* species. As with most response regulators, MtrA activity is controlled through phosphorylation and dephosphorylation by its cognate sensor kinase, MtrB. Our data, and previously published work on MtrA in mycobacteria and corynebacteria, support a model in which removing MtrB results in constitutive phosphorylation and activation of MtrA which then modulates the expression of its target genes in the cell. In *S. coelicolor* and *S. venezuelae* these include key developmental genes and there is a significant overlap with the regulon controlled by the master repressor of development, BldD, which is activated for DNA binding by the secondary messenger cyclic-di-GMP (Tschowri et al. 2014). Exactly where

MtrA fits in to the hierarchy of developmental regulation is not yet clear and there is much work to be done to determine the role of the MtrAB two-component system in the cell biology of *Streptomyces* species. However, our data show that MtrA has an essential role in *S. venezuelae* and shares some functions with *M. tuberculosis* MtrA. It will be interesting to compare the complete overlap when the TB-MtrA regulon is published (Purushotham et al. 2015). Loss of MtrB has minor effects on growth and sporulation in *S. venezuelae* and it will be interesting to deplete MtrA activity in future studies to examine the effects on growth, development and secondary metabolite production. Here we have focused on the role of MtrA in regulating secondary metabolism and how it might be exploited to activate cryptic BGCs. The effects on antibiotic production appear to be direct in both *S. coelicolor* and *S. venezuelae* because MtrA activates expression of the chloramphenicol transporter CmlN and activation of MtrA switches on chloramphenicol production. It also binds to the promoters of the cluster specific activators ActII-4 and RedZ in *S. coelicolor* and activates the products under their control, actinorhodin and undecylprodigiosin, respectively. In a developmental time course it is remarkable that MtrA binds to sites spanning 27 of the 31 BGCs in *S. venezuelae* and directly affects the expression of target genes in at least 22 of these BGCs. In vegetatively growing *S. coelicolor* MtrA binds to sites spanning 21 out of 29 BGCs. We propose that MtrA is a master regulator of secondary metabolism in *Streptomyces* species. Consistent with this, unbiased metabolomics analysis of *S. venezuelae* shows that deleting *mtrB* results in a global change in the metabolome. Given that secondary metabolite BGCs are often subject to complex and multilevel regulation, and given the energy costs associated with making these natural products, it is perhaps not surprising that we do not see obvious over-production of other compounds in the Δ*mtrB* strains. We predict that deletion of known BGCs in the Δ*mtrB* mutants and / or depletion of *mtrA* transcripts will have positive effects on the production of additional secondary metabolites.

In summary, our study has revealed an important role for MtrA, a previously uncharacterised but essential response regulator, in the life cycles of streptomycetes. Significant work remains to be done to elucidate its role in *Streptomyces* species but our data

suggest it might play a similar role to CtrA in *Caulobacter crescentus*, acting as a master regulator of the cell cycle alongside other master regulators such as BldD. Perhaps even more important is the fact that it coordinates production of antibiotics and other natural products with the developmental programme. Rapid progress has been made recently in elucidating the roles of such developmental regulators, using next generation sequencing techniques and *S. venezuelae* as a model system (Bush et al. 2015). We have shown that *S. venezuelae* can also be used as a model to study global effects on secondary metabolism and to elucidate the regulatory cascades that link secondary metabolism and differentiation. Understanding these genetic circuits is essential if we are to unlock the full potential of these bacteria and their secondary metabolites.

Materials and Methods

Strains, plasmids and primers. The bacterial strains, plasmids and cosmids and primers used in this study are listed in Tables S6-8. The Sv- and TB-MtrA expression vectors have been deposited with AddGene (ID 85988-94). *S. venezuelae* NRRL B-65442 is deposited in the USDA Agricultural Research Services (ARS) Culture Collection (http://nrrl.ncaur.usda.gov/cgi-bin/usda/prokaryote/report.html?nrrlcodes=B%2d65442). Plasmid stocks were prepared using Qiagen miniprep kits and cosmids were prepared as described previously (Kieser et al. 2000). Genes were deleted using the ReDirect PCR targeting method (Gust et al. 2003) and an *S. venezuelae* NRRL B-65442 cosmid library provided by Professor Mark Buttner at the John Innes Centre, Norwich. All of the expression constructs used in this work were made by Genscript by synthesising the *mtrA* alleles with or without 3' tags and then cloning into the required vectors. Liquid cultures of *E. coli* were routinely grown shaking at 220 rpm in Lennox Broth at 37°C. Liquid cultures of *S. coelicolor* or *S. venezuelae* were grown in Mannitol Yeast Extract Malt Extract (MYM) at 30°C, shaking at 220 rpm. Cultures grown on solid MYM agar were grown at 30°C. Spore stocks of *S. coelicolor* were prepared from cultures grown on Soya Flour + Mannitol (SFM) agar. All media recipes have been published previously and *Streptomyces* spores were prepared as

described (Kieser et al. 2000). To determine the developmental growth in liquid culture *S. venezuelae* and mutant strains were grown, shaking in 35ml MYM in 250ml conical flasks containing springs at 30°C at 220rpm. A spore inoculum sufficient to reach an OD_{600} of 0.35 after 8 hours of growth was added to 35ml of MYM media in 250 ml flasks containing springs. The culture densities were measured at OD_{600}. Development in liquid cultures was monitored using an GXML3000B microscope from GX optical. Pictures of agar plate grown colonies were taken with a Zeiss SVII stereo microscope. SEM images were taken at the bioimaging facility at the John Innes Centre.

Purification of MtrA-His. The *mtrA* gene was cloned into pETDuet1 (Novagene) to express the protein with a C-terminal hexa-His tag and purified using a batch method. Cell pellets were resuspended in 25ml lysis buffer (75mM Tris-HCl pH8, 20mM NaCl, 0.1% Triton X100, 50 µl 10mg/ml lysozyme, 3 x Pierce EDTA-free Protease Inhibitor Mini Tablets (Thermo Scientific) in 1L) and incubated for 30 minutes at room temperature. The cell lysate was sonicated 2 x 40 seconds at 50Hz with 1 minute in between sonication steps. Cell debris was removed by centrifugation at 18,000rpm for 20 minutes at 4°C in Beckman Coulter Avanti® J-20 high performance centrifuge using a JLA-25-50 rotor (Beckman Coulter). The supernatant was transferred in a fresh 50ml Falcon tube and 350µl of Ni-NTA agarose beads (Qiagen) were added and incubated under gentle agitation for 1 hour at 4°C. The Ni-NTA agarose beads were spun down gently (maximum of 1200rpm, 4°C) and the supernatant was discarded. The Ni-NTA beads were resuspended in 2ml wash buffer (75mM Tris-HCl pH8, 200mM NaCl, 10% glycerol, 10mM $MgCl_2$, 0.1mM DTT, 20mM 2-mercaptoethanol in 1L) and transferred in Polypropylene columns (1ml, Qiagen). The beads in the column were washed with 20ml wash buffer. The protein was eluted from the beads with 2.5ml elution buffer (wash buffer plus 350mL imidazole in 1L). Polyclonal antiserum was raised by Cambridge Research Biochemicals. Immunoblotting was performed as described previously (Hutchings, Hong, Leibovitz, et al. 2006).

DNA binding studies. Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) was performed on *S. coelicolor* M145 Δ*mtrA* + MtrA-3xFlag and *S. venezuelae* NRRL B-65442 Δ*mtrA* + MtrA-3xFlag grown in liquid MYM medium (Kieser et al. 2000). Note that *S. coelicolor* M145 grows vegetatively and does not differentiate under these growth conditions whereas *S. venezuelae* NRRL B-65442 undergoes a full life cycle in 20 hours. Samples were taken at 16 and 20 hours for *S. coelicolor* and at 8, 10, 12, 14, 16, 18 and 20 hours for *S. venezuelae*. ChIP-seq and electrophoretic mobility shift assays (EMSAs) were performed as described previously (Crack et al. 2015) using probes generated by PCR with 6-Fam labelled primers from Integrated DNA Technologies (Table S8). ChIP-seq Gels were visualised using a Typhoon FLA 9500 laser scanner (GE Healthcare) with LBP/BPB1 emission filter, Exmax 495nm Emmax 576nm, at 50μM resolution. ChIP-seq data was analysed as described previously (Bush et al. 2013) and peaks were visually inspected using integrated genome browser (Nicol et al. 2009). Binding sites were identified using MEME (Bailey et al. 2015).

RNA-sequencing. Duplicate wild-type and Δ*mtrB* cultures were grown for 14 and 20 hours in MYM medium and RNA was prepared as described previously (Munnoch et al. 2016). Libraries were prepared and sequenced by Vertis Biotechnologie and analysed using CLC Genomics Workbench.

LCMS and PCA.

Analytical HPLC was performed on an HPLC 1100 system (Agilent Technologies) connected to a Gemini® 3 μm NX-C18 110 Å, 150×4.6 mm column (Phenomenex) and on a Synergi™ 4 μm Fusion-RP 80 Å LC column 250×10 mm. UPLC-MS for metabolic profiling was performed on a Nexera X2 liquid chromatograph (LC-30AD) LCMS system (Shimadzu) connected to an autosampler (SIL-30AC), a Prominence column oven (CTO-20AC) and a Prominence photo diode array detector (SPD-M20A). A Kinetex® 2.6 μm C18 100 Å, 100×2.1 mm column (Phenomenex) was used. The UPLC-System was connected with a LCMS-IT-TOF Liquid Chromatograph mass spectrometer (Shimadzu). UPLC-HRMS Data was acquired on an

Acquity UPLC system (Waters Corporation) equipped with an ACQUITY UPLC® BEH 1.7 µm C18, 1.0 × 100 mm column (Waters Corporation) and connected to a Synapt G2-Si high resolution mass spectrometer (Waters Corporation). A gradient between mobile phase A (H_2O with 0.1 % formic acid) and mobile phase B (acetonitrile with 0.1 % formic acid) at a flow rate of 80 µL/min was used. Initial conditions were 1 % B for 1 min, ramped to 90 % B within 6 minutes, ramped to 100 % B within 0.5 min, held for 0.5 min, returned to 1 % B within 0.1 min and held for 1.9 min. MS spectra were acquired with a scan time of one second in the range of *m*/*z* = 150 - 1200 in positive MSe-Resolution mode. The following instrument parameters were used: capillary voltage of 3 kV, sampling Cone 40, source offset: 80, source temperature of 120 °C, desolvation temperature 350 °C, desolvation gas flow 800 L/h. A solution of sodium formate was used for calibration. A solution of leucine encephalin (H_2O/MeOH/formic acid: 49.95/49.95/0.1) was used as lock mass and was injected every 15 sec. The lock mass has been acquired with a scan time of 0.3 sec and 3 scans were averaged each time. The lock mass (*m*/*z* = 556.2766) has been applied during data acquisition.For processing metabolomics data we used the Software Progenesis QI (Waters). All solvents for analytical HPLC and UPLC-MS were obtained commercially at least in HPLC grade from Fisher Scientific and were filtered prior to use. Formic acid (EZInfo 3.0 (MKS Umetrics AB) was used for plotting PCA data.

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597

598 **Author contributions**

599 FK and RFS constructed the *S. coelicolor* mutants and FK undertook phenotype analysis, NS
600 constructed all the *S. venezuelae* strains and constructs, analysed phenotypes and performed
601 ChIP-seq experiments, JTM performed RNA-seq and dRNA-seq experiments, JTM and GC
602 analysed the ChIP- and RNA-seq datasets, NH made constructs and prepared strain extracts
603 for LCMS, DH purified and analysed all natural products including LCMS and PCA analyses,
604 PAH, MIH and BW conceived the study and all the authors analysed data and wrote the
605 manuscript.

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