1 **MtrA is an essential regulator that coordinates antibiotic production**

2 **and sporulation in _Streptomyces_ species**

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25   **Abstract.** *Streptomyces* bacteria make numerous secondary metabolites, including half of all

26   known antibiotics. Understanding the global regulation of secondary metabolism is important

27   because most *Streptomyces* natural products are not made under laboratory conditions and

28   unlocking 'cryptic' biosynthetic gene clusters (BGCs) is a major focus for natural product

29   discovery. Production is coordinated with sporulation but the regulators that coordinate

30   development with antibiotic biosynthesis are largely unknown. Here we characterise a highly

31   conserved actinobacterial response regulator called MtrA in antibiotic-producing

32   *Streptomyces* species. We show that MtrA is an essential global regulator of secondary

33   metabolism that directly activates antibiotic production in in *S. coelicolor* and *S. venezuelae*.

34   MtrA also controls key developmental genes required for DNA replication and cell division and

35   we propose that MtrA is the missing link that coordinates secondary metabolism with

36   development in *Streptomyces* species.

37

38   **Introduction.**

39   *Streptomyces* secondary metabolites account for two thirds of all known antibiotics and

40   numerous other compounds that are used in human medicine as anticancer, anti-parasitic,

41   antiviral and immunosuppressant drugs. Discovery of these natural products peaked in the

42   1950s but there has been a resurgence of interest in the 21st century, driven by genome

43   sequencing and the increasing threat of drug resistant infections [1]. Despite their importance

44   however, we still have a poor understanding of how *Streptomyces* bacteria control the

45   production of their secondary metabolites. This is important because ≥75% of their secondary

46   metabolite biosynthetic gene clusters (BGCs) are not expressed in laboratory culture and

47   activating cryptic BGCs could facilitate the discovery of new antibiotics and other useful

48   compounds [2,3].

49   The major way in which bacteria sense and respond to their environment is through

50   two-component systems and several have been implicated in the regulation of antibiotic

51   production in *Streptomyces* species [4]. Two component systems typically consist of a

52   bifunctional sensor kinase and a cognate response regulator [5]. The sensor kinase perceives

53   an extracellular signal and activates its cognate response regulator through a two-step

54   phosphorylation. The phosphorylated regulator (RR~P) brings about a response to the original

55   signal, usually by modulating target gene expression. In the absence of signal, the bifunctional

56   sensor kinase dephosphorylates its response regulator to keep the response switched off [6].

57   The delicate balance of kinase and phosphatase activities is crucial in modulating the activity

58   of the response regulator and its target genes during the bacterial cell cycle [5]. Cross-talk

59   between systems is rare in wild-type cells but removal of a sensor kinase can result in

60   constitutive activation of its response regulator by a non-cognate sensor kinase or by the

61   cellular pool of acetyl phosphate [7]. Similarly, altering a sensor kinase to block its phosphatase

62   activity might result in a response regulator that cannot be dephosphorylated and is

63   constitutively active [5].

64   Here we report characterisation of the highly conserved actinobacterial response

65   regulator MtrA in the model organism *Streptomyces venezuelae* [8]. MtrA was first identified as

66   an essential regulator in *Mycobacterium tuberculosis* (Mycobacterium tuberculosis regulator

67   A) [9]. *M. tuberculosis* MtrA (TB-MtrA) regulates the expression of *dnaA* and *dnaN*, which are

68   essential for DNA replication, and sequesters the origin of DNA replication, *oriC*, in dividing

69   cells [10]. TB-MtrA also regulates the expression of cell division genes and interacts directly with

70   the DnaA protein [10]. MtrA is activated when the MtrB sensor kinase localises to the site of cell

71   division through interaction with FtsI and DivIVA. These data have led to a model in which

72   oscillations in TB-MtrA~P levels play a key role in cell cycle progression by repressing DNA

73   replication and activating cell division [10-12]. In the closely related *Corynebacterium glutamicum*,

74   Cg-MtrA controls genes involved in cell wall remodelling and the osmotic stress response [13,14]

75   but deletion of the *mtrAB* genes gives rise to elongated cells which are indicative of a defect

76   in cell division [15].

77   *Streptomyces* bacteria are filamentous saprophytes which grow through the soil as

78   branching substrate mycelium that extends at the hyphal tips. Nutrient starvation triggers the

79   production of reproductive aerial hyphae that accelerate DNA replication, generating up to 200

80   copies of the chromosome in each aerial hypha, before undergoing cell division to form chains

81   of unigenomic spores. Aerial hyphae production and sporulation are coordinated with the

82   production of bioactive secondary metabolites, including antibiotics. *S. coelicolor* has

83   traditionally been used to study development and antibiotic production because it makes

84   pigmented antibiotics and spores but it only differentiates into aerial hyphae and spores on

85   solid agar. *S. venezuelae* has emerged as an alternative model because it completes a full

86   developmental life cycle in liquid growth medium in ~20 hours [16]. Here we report that MtrA is

87   essential for the growth of *S. venezuelae* and directly regulates the expression of genes

88   involved in DNA replication, cell division and secondary metabolism. ChIP-seq throughout the

89   *S. venezuelae* life-cycle shows that Sv-MtrA activity displays a biphasic plasticity such that it

90   is active during vegetative growth and sporulation, but inactive during formation of aerial

91   hyphae. We propose that in *Streptomyces* species, MtrA represses DNA replication and cell

92   division during active growth and following sporulation. We also show that Sv-MtrA binds to

93   sites spanning ~85% of the BGCs in *S. venezuelae* and directly modulates expression of

94   target genes in at least 75% of these. ChIP-seq in *S. coelicolor* yielded similar results and key

95   developmental genes bound by MtrA in both streptomycetes include *bldM, ftsZ, ssgA, smc,*

96   *smeA, whiI, whiB, whiD* and *wblE.* We propose that MtrA is an essential master regulator that

97   coordinates differentiation and secondary metabolism in *Streptomyces* species.

98

99   **Results**

100   **MtrA is essential in *Streptomyces venezuelae*.**

101   To investigate the function of the MtrAB two component system in *S. venezuelae* we deleted

102   either *mtrA* or *mtrB* [17]. *mtrB* was deleted easily but all attempts to delete *mtrA* were

103   unsuccessful until we introduced a second copy of *mtrA in trans* suggesting MtrA is essential

104   in *S. venezuelae.* It follows that MtrA must be active in the absence of MtrB, otherwise deleting

105   *mtrB* would be lethal. We propose that deletion of *mtrB* leads to constitutive phosphorylation

106   of MtrA, either by the cellular pool of acetyl phosphate or another sensor kinase. We observed

107   a similar result with *Streptomyces coelicolor* VanRS, where VanR and its target genes are

108 constitutively active in a ∆vanS mutant [7]. Surprisingly, deletion of *mtrB* has no effect on the

109 growth rate of *S. venezuelae* in liquid medium (Figure S1).

110

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

112 Microarray data [18] shows that *mtrA* expression levels remain fairly constant during the *S.*

113 *venezuelae* life cycle and we do not detect any significant change in MtrA protein levels during

114 the life cycle using immunoblotting (Figure S2). Attempts to compare levels of phosphorylated

115 and non-phosphorylated MtrA using Phostag were unsuccessful and so, to examine MtrA

116 activity during the *S. venezuelae* life cycle, we performed ChIP-seq at two-hour time points (8-

117 20 hours) from active growth through to sporulation (accession number GSE84311, Table S1).

118 We expressed MtrA-3xFlag *in trans* under the control of its own promoter and deleted the

119 native *mtrA* gene. Survival of this strain shows that MtrA-3xFlag is functional and there is no

120 significant difference in growth rate between this strain and the wild-type (Figure S1). Analysis

121 of the ChIP-seq data using a *P* value > 0.05 revealed that only one target is enriched at all

122 time points, the promoter region of the *ectABCD* operon, which is the BGC for the secondary

123 metabolites ectoine and 5' hydroxyectoine (5HE). We do not detect ectoine or 5HE in the wild-

124 type or ∆*mtrB* mutant using LCMS with authentic standards so we predict that MtrA represses

125 *ectABCD* expression throughout the life cycle. Analysis of all the MtrA ChIP-seq datasets

126 using *P* > 0.05 shows that the pattern of binding of MtrA is dynamic throughout the lifecycle

127 with peak DNA binding activity seen at 10 and 20 hours, coinciding with mid-exponential

128 growth, and at 20 hours, in spores (Figure S3). Mapping ChIP-seq reads at targets such as

129 the *mtrA* promoter provides a good illustration of this biphasic activity (Figure 1). Differential

130 RNA sequencing shows that the major *mtrAB-lpqB* transcript is leaderless, with an MtrA

131 binding site immediately upstream of the -35 site, suggesting positive autoregulation. A minor

132 transcript starts at -79bp suggesting two promoters might drive expression of the *mtrAB-lpqB*
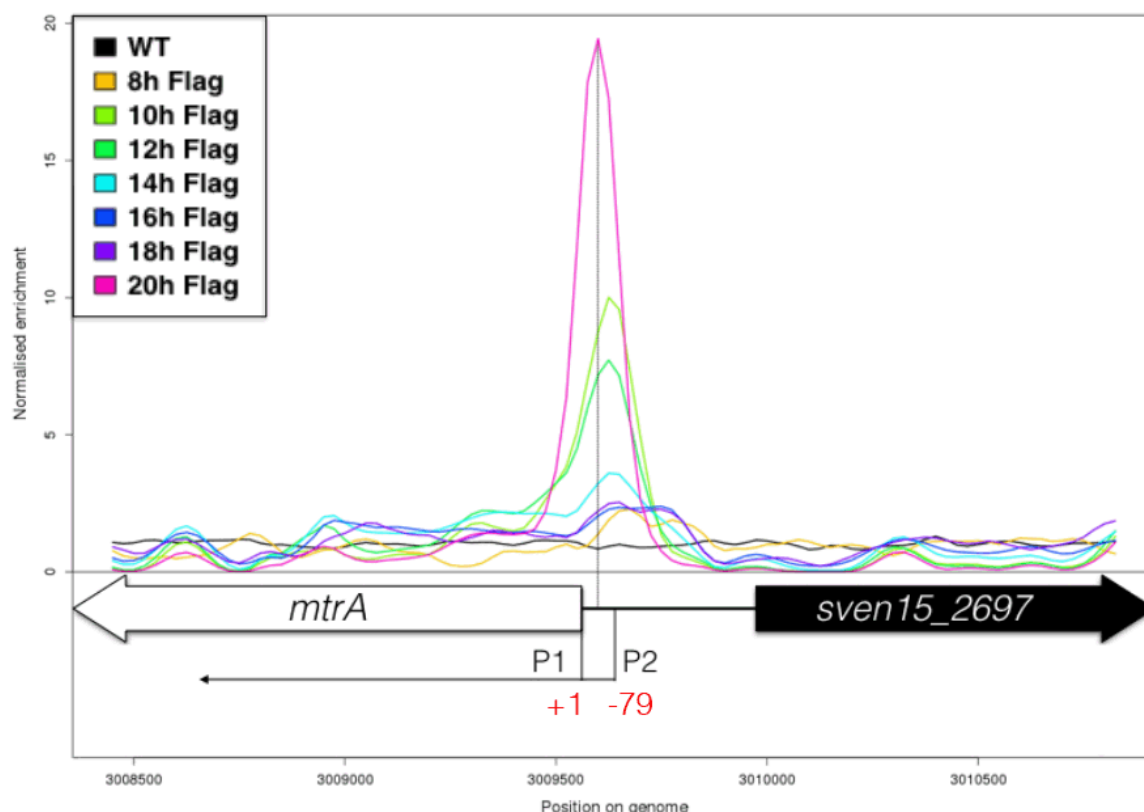
133 operon (Figure 1).

134

135

Figure 1. MtrA autoregulates its own expression. *ChIP-seq during the S. venezuelae life cycle shows that MtrA has highest activity in actively growing mycelium (10 and 12 hours) and spores (20 hours). The peaks shown are at the mtrA promoter, which has two transcript start sites at +1 (P1) and -79 (P2). Deletion of mtrB increases mtrA transcript levels ~3-fold indicating positive autoregulation.*

136

137 **Identifying the MtrA binding site.** To identify an MtrA consensus binding sequence we used

138 MEME [19] to analyse three MtrA target promoters identified by ChIP-seq and confirmed *in vitro*

139 using electrophoretic mobility shift assays (EMSAs). This analysis identified an AT-rich 7 bp

140 motif present at all three promoters (Figure 2). We then used MEME to analyse 50 bp of

141 sequence from beneath each peak at the 14h and the 16/18h time points and this analysis

142 identified a conserved sequence at each target which is effectively a direct repeat of the AT

143 rich motif (Figure 2). However, many of the sequences enriched in the MtrA ChIP-seq dataset

144 do not obviously contain this motif and we hypothesise that MtrA may interact with other

145 transcription factors. Immunoprecipitation experiments using anti-Flag beads pulled down four

146 different regulators in the MtrA-3xFlag cultures that are not immunoprecipitated in the wild-

147 type control. The upstream regions of the genes encoding these regulators (*sven15_0243*,

6

148   *sven15_2691*, *sven15_3571* and *sven15_4644*) are all bound by MtrA (Table S1).

149   Sven15_3571 is DnaA, which initiates DNA replication and acts as a transcription factor in

150   bacteria, and is also bound *in vivo* by TB-MtrA {Purushotham:2015iq}. Thus, our data suggests

151   (but does not prove) that MtrA forms complexes with other DNA binding proteins and this could

152   explain why there are so many enrichment peaks in the 20-hour ChIP-seq dataset. Interaction

153   between regulatory proteins to form heterodimers is not unprecedented in *S. venezuelae.* The

154   response regulator BldM modulates one set of target genes as a homodimer and another set

155   by forming heterodimers with the WhiI response regulator [20]. The developmental regulators

156   WhiA and WhiB are also proposed to interact and have identical ChIP-seq regulons [21].

157

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

159   ChIP-seq shows that MtrA activity changes during the *S. venezuelae* life cycle (Figure 1) and

160   RNA sequencing of wild-type and Δ*mtrB* strains shows that expression of *mtrA* is 3-fold

161   upregulated in the Δ*mtrB* mutant (Table S2) which is consistent with activation by MtrA~P.

162   Given the essentiality of MtrA and its altered activities at different stages of the life cycle we

163   predicted that it could play a key role in regulating cell cycle progression in *S. venezuelae*.

164   Indeed, ChIP-seq shows that many key developmental genes are bound by MtrA in *S.*

165   *venezuelae* and *S. coelicolor* (see Tables S1 and S2 for complete lists). Remarkably, *M.*

166   *tuberculosis* and *S. venezuelae* MtrA proteins share some common targets, including the

167   promoter regions of *wblE*, *dnaA* and *dnaN*, and the *oriC* region between *dnaA* and *dnaN*

168   (Figure 3). RNA-seq shows that deletion of *mtrB* does not affect the expression levels of *dnaA*

169   or *dnaN* under the conditions we used but deletion of *mtrB* causes a two-fold increase in *wblE*

170   transcript levels, suggesting MtrA-dependent activation. MtrA also directly represses *adpA*

171   expression, which is down-regulated nearly 4-fold in the Δ*mtrB* mutant (Table S2).

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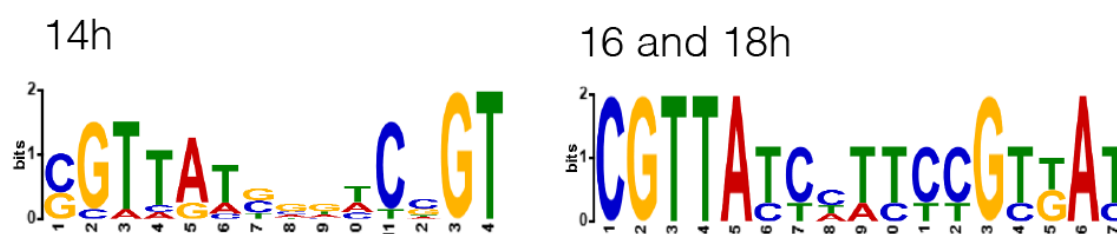| Target	P value	Binding site	
Sven15_0205 (ectA)	4.23e-5	GCGTTACCCATCCGTTATAAACGGTCAT	
Sven15_0880 (cmlF)	4.23e-5	ATTCGCCATCTACGTTCTCGGGCTGGCC	
Sven15_3571 (dnaA)	1.51e-5	CACAATCTTTTCCGTTCTGTCCTTACCT	

173

 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.*

174

175   AdpA controls key developmental genes and represses DNA replication in *Streptomyces*

176   species [22,23]. *wblE* encodes a WhiB-like transcription factor of unknown function that appears

177   to be essential [24]. Sv-MtrA also binds upstream of the cell division genes *ssgA* and *ssgB*,

178   which are upregulated in the Δ*mtrB* mutant, and the *ftsZ* gene, which is unaffected by loss of

179   MtrB, probably because this gene is subject to complex regulation (Figure 3B and Table S2).

180   SsgAB localise the divisome marker protein FtsZ to the correct positions in aerial hyphae to

181   mark the sites of cell division, prior to sporulation [25,26]. Sv-MtrA also regulates expression of

182   the *smeA-ssfA* operon, which is elevated between 2- and 4-fold in the Δ*mtrB* mutant. SmeA

183   targets the DNA pump SffA to the cell division septa [16]. MtrA also binds upstream of (and

184   activates some) key developmental regulators, including *whiB*, *whiD*, *whiI* and *bldM* (Table

185   S2) [20,21,27].

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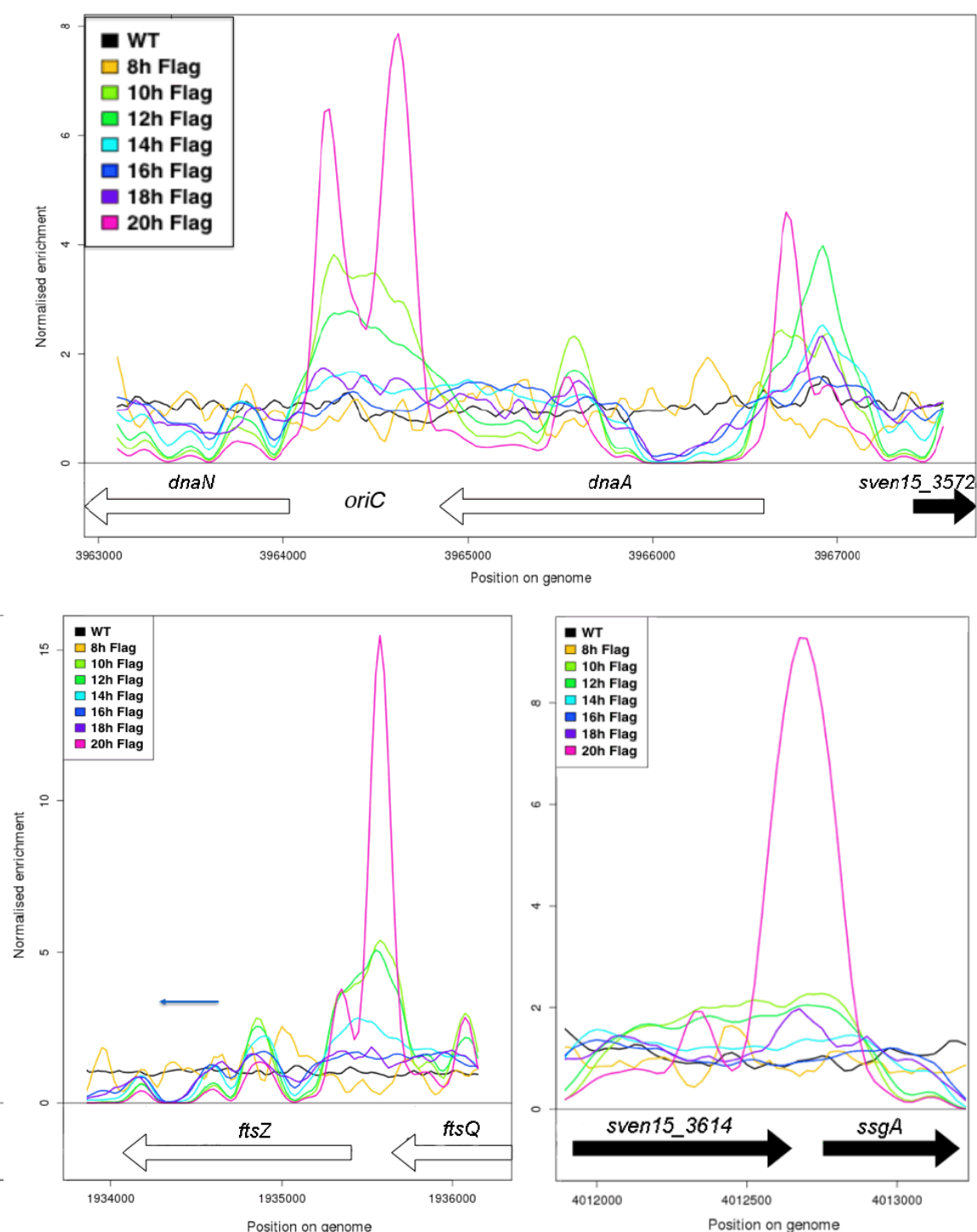
188

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.*

189

190   Many of the Sv-MtrA target genes are also repressed by BldD (in complex with c-di-GMP).

191   Sv-MtrA does not regulate *bldD* expression but it does regulate the expression of genes

192   encoding proteins that metabolise cyclic di-GMP [28] (Table S2).

193

194   **MtrA regulates global BGC expression in *Streptomyces venezuelae*.**

195   ChIP-seq shows that MtrA binds to sites spanning 28 of the 31 BGCs predicted by antiSMASH

196   3.0 in the *S. venezuelae* genome [29] (Table S3). The only BGCs with no MtrA enrichment are

197   those encoding biosynthesis of the desferrioxamine siderophores, the WhiE polyketide spore

198   pigment and an unknown natural product. Of these three clusters, the WhiE BGC is

199   upregulated in the Δ*mtrB* mutant suggesting indirect regulation by MtrA, probably via BldM [20].

200   The other two BGCs are unaffected by loss of MtrB (Table S3). Of the 28 BGCs that are bound

201   by MtrA, nine have genes that are positively regulated by MtrA, 10 have genes that are

202   negatively regulated by MtrA and three are subject to both positive and negative regulation by

203   MtrA at individual genes within the gene cluster (Table S3). The other six BGCs have sites

204   that are enriched in MtrA ChIP-seq but expression of the genes nearest the ChIP-seq peaks

205   are not affected by deletion of *mtrB* under the conditions we used (Table S3). We predict that

206   one of these, the *ectABCD* operon, is repressed by MtrA because the *ectA* promoter is bound

207   by MtrA at all time points. Consistent with this, we cannot detect ectoine or 5HE in the wild-

208   type or Δ*mtrB* strains. Since most of the *S. venezuelae* BGCs are uncharacterised we know

209   little about their cluster specific regulation, or their products. The antiSMASH predictions we

210   have used here are also likely to include additional flanking genes that may not be part of the

211   BGC so the actual number of BGCs bound by Sv-MtrA may be slightly lower.

212

213   **MtrA activates chloramphenicol production in *S. venezuelae*.**

214   Phenotypic screening of the Δ*mtrB* mutant revealed that a cryptic antibacterial is activated by

215   removing MtrB but not by simply over-expressing *mtrA* in wild-type *S. venezuelae* (Figure S4).

10

216 This is probably because MtrA~P levels only increase in the absence of MtrB. *S. venezuelae*

217 encodes the biosynthetic pathway for the antibiotic chloramphenicol, which according to

218 previous studies is silent in the wild-type strain [30]. ChIP-seq shows that Sv-MtrA binds in the

219 intergenic region between the divergent *cmlF* (*sven15_0879*) and *cmlN* (*sven15_0880*) genes

220 (Figure 4) and expression of *cmlN* is 6-fold upregulated in the Δ*mtrB* mutant, consistent with

221 direct activation by MtrA. CmlN is an efflux permease, predicted to export chloramphenicol [30].

222 HPLC confirmed that chloramphenicol is produced in the Δ*mtrB* mutant but we also detected

223 very low levels in the wild-type strain suggesting the cluster is not completely silent (Figure 4).

224 Cultivation for 24 hours in biological and technical triplicates confirmed an increased

225 production of chloramphenicol in the Δ*mtrB* mutant with a mean concentration of 0.41 mg/L

226 which is >30 times higher than the wild-type strain (0.013 mg/L) or wild-type over-expressing

227 *mtrA* (0.010 mg/L).

228

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

230 While loss of MtrB clearly leads to increased production of chloramphenicol, we were

231 interested to know if there are any other effects on the metabolome. We cultivated the wild

232 type strain and three independently isolated *ΔmtrB* mutants in biological and technical

233 triplicates and analysed the extracts by UPLC/HRMS using an untargeted metabolomics

234 approach. Runs were aligned to compensate for between-run variation and a peak-picking

235 algorithm was applied to allow for the immaculate matching of each feature (a discrete *m/z*

236 value and its retention time) among all runs. Following normalisation, features could be

237 compared quantitatively and their putative identity proposed based on their high-resolution

238 MS-signal. Comparing the level of metabolite signals, it appeared that all *ΔmtrB* mutants

239 showed increased production of a considerable portion of their putative secondary

240 metabolites. To display multidimensional data, we used Principle Component Analysis (PCA)
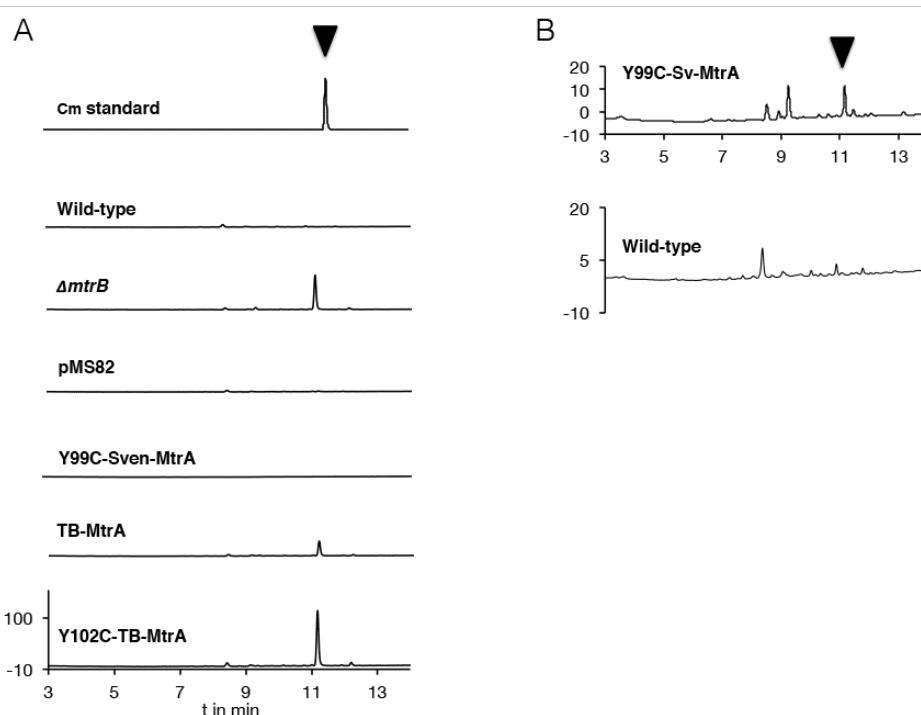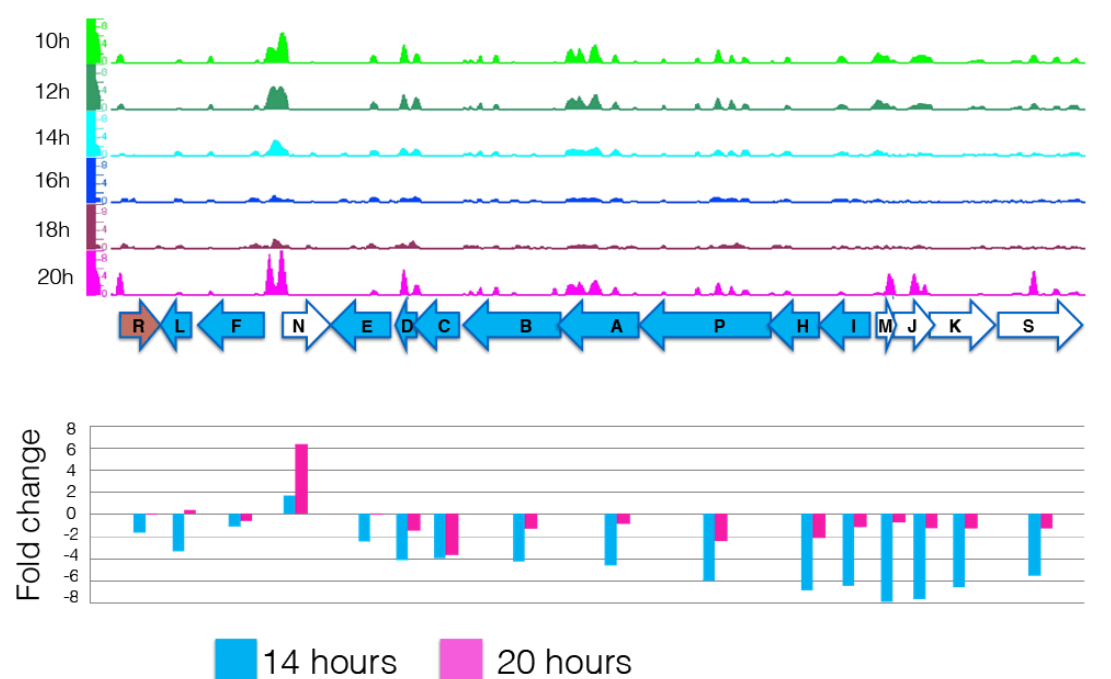
241 (Figure 5).

242

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 ∆mtrB strains at 14 and 20 hours shows cmlF is 6-fold upregulated in ∆mtrB 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, ∆mtrB, 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.*

243   Each sphere in the 3D Plot represents one dataset obtained from a particular UPLC-HRMS

244   run. Data from the *ΔmtrB* mutant strains clearly group together, and are distinct from data

245   obtained from the wild type, while variations within each group are comparably small. The 3D

246   Plot therefore shows consistent and global changes in the metabolome upon loss of MtrB
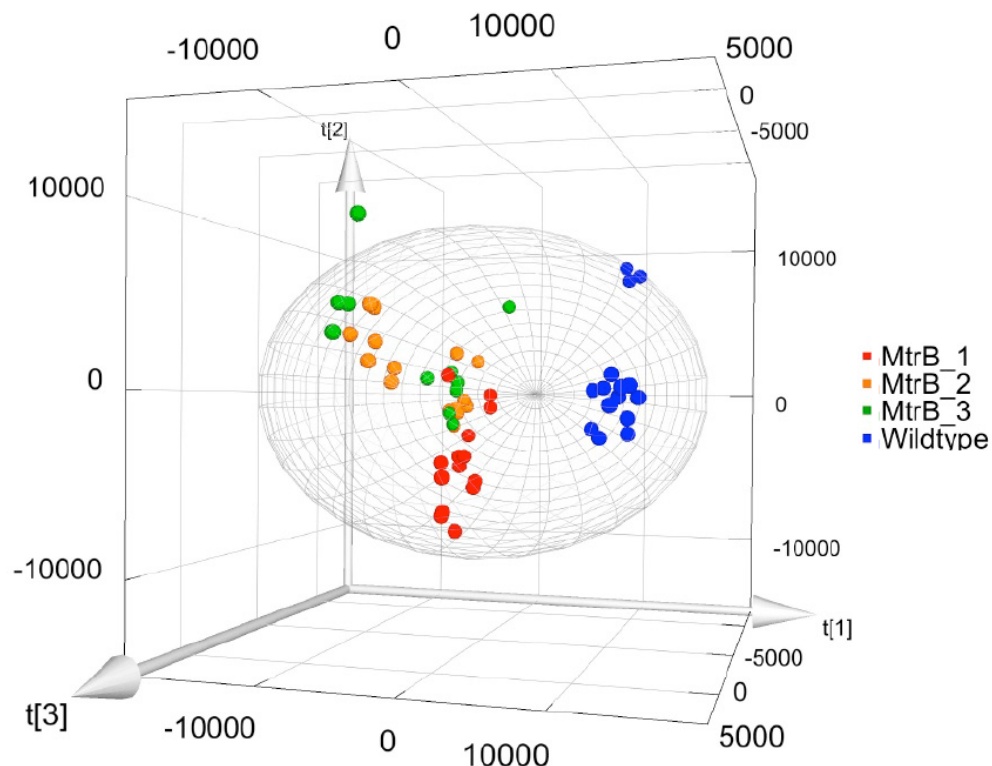
247   (Figure 5).

248

249

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.

250

MtrA directly activates antibiotic production in *Streptomyces coelicolor.*

252   MtrA is conserved in all sequenced *Streptomyces* genomes so we reasoned that it might

253   activate antibiotic production in other streptomycetes. To test this, we deleted *mtrB* in the

254   model organism *Streptomyces coelicolor* [31]. The 16S rDNA phylogenetic tree of the family

255   *Streptomycetaceae* shows that *S. venezuelae* (clade 40) is highly divergent from *S. coelicolor*

13

256   (clade 112) so we reasoned that if deleting *mtrB* switches on antibiotic production in these

257   distantly related species it is probably universal to all streptomycetes [32]. The *S. coelicolor*

258   Δ*mtrB* mutant over-produces the red antibiotic undecylprodigiosin and the blue antibiotic

259   actinorhodin and we also detected significant amounts of metacycloprodigiosin, a potent

260   anticancer compound encoded by the undecylprodigiosin BGC but not previously reported

261   from *S. coelicolor* (Figure 6). Other secondary metabolites are clearly down-regulated in the

262   Δ*mtrB* mutant, including the siderophores desferrioxamine B and E and germicidin A. We

263   performed ChIP-seq in *S. coelicolor* because its BGCs and their natural products are the most

264   well defined of any *Streptomyces* species [31,33,34]. The results show that MtrA binds to sites

265   spanning 21 of its 29 BGCs (Tables S4 and S5). MtrA binds upstream of the genes encoding

266   the actinorhodin activator ActII-4 and the undecylprodigiosin activator RedZ (Figure S11).

267   MtrA does not bind to the desferrioxamine BGC (*desABCD*) in *S. coelicolor* (Table S4) but

268   does bind upstream of *sco4394*, which encodes DesR, an iron dependent repressor of

269   desferrioxamine biosynthesis (Figure S5). A single type III polyketide gene, *SCO7221*, is

270   responsible for germicidin biosynthesis [35] and it is not regulated by MtrA, so the effect on

271   germicidin biosynthesis must be indirect.

272

273   **TB-MtrA activates chloramphenicol production in *S. venezuelae*.**

274   Modulating MtrA activity by deleting *mtrB* is time consuming and impractical when applied to

275   multiple *Streptomyces* strains. Instead we tested whether alternative, gain-of-function MtrA

276   proteins might be used to activate antibiotic production. We constructed expression vectors

277   for wild-type TB-MtrA, gain-of-function Y102C TB-MtrA [36] and a Y99C Sv-MtrA variant which

278   has the equivalent change to Y102C TB-MtrA (Figure S6). We were unable to delete the native

279   *mtrA* gene in strains carrying these constructs but all three MtrA constructs induced

280   chloramphenicol production in wild-type *S. venezuelae* (Figure 4). Y99C Sv-MtrA switches on

281   the production of relatively small amounts of chloramphenicol in wild-type *S. venezuelae* but

282   the TB-MtrA proteins switch on much higher levels of production, with the strain expressing

283   Y102C-TB-MtrA making more chloramphenicol than the Δ*mtrB* mutant (Figure 4). These

284   results suggest that expressing different MtrA proteins might be a useful avenue to explore in

285   terms of activating BGCs in *Streptomyces* species and our constructs are available from
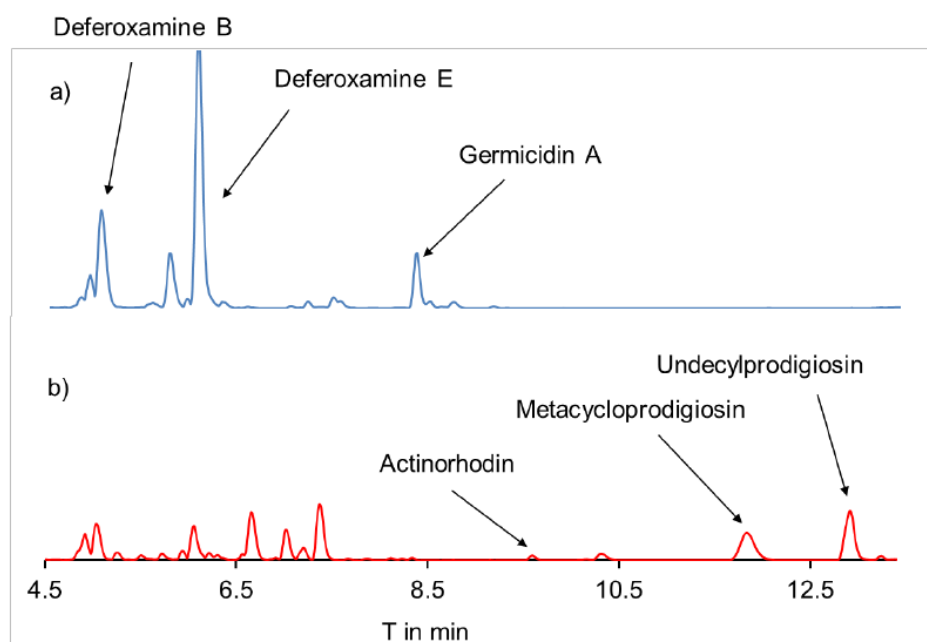
286   AddGene (IDs 85988-94).

287

288

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.*

289

290   **Discussion.**

291   Our data, and previously published work on MtrA in mycobacteria and corynebacteria, support

292   a model in which removing MtrB results in constitutive phosphorylation and activation of MtrA

293   which then constitutively activates or represses its target genes in the cell. In *S. coelicolor* and

294   *S. venezuelae* these include key developmental genes and there is a significant overlap with

295   the regulon controlled by the master repressor of development, BldD, which is activated for

296   DNA binding by the secondary messenger cyclic-di-GMP [28]. Exactly where MtrA fits in to the

297   hierarchy of developmental regulation is not yet clear but MtrA has an essential role in *S.*

298   *venezuelae* and shares some functions with *M. tuberculosis* MtrA [10]. Here we have focused

15

299   on the role of MtrA in regulating secondary metabolism and how it might be exploited to

300   activate cryptic BGCs. The effects on antibiotic production appear to be direct in both *S.*

301   *coelicolor* and *S. venezuelae* because MtrA activates expression of the chloramphenicol

302   transporter CmlN and switches on chloramphenicol production. It also binds to the promoters

303   of the cluster specific activators ActII-4 and RedZ in *S. coelicolor* and activates the products

304   under their control, actinorhodin and undecylprodigiosin. In a developmental time course it is

305   remarkable that MtrA binds to sites spanning 27 of the 31 BGCs in *S. venezuelae* and directly

306   affects the expression of target genes in at least 22 of these BGCs. In vegetatively growing *S.*

307   *coelicolor* MtrA binds to sites spanning 21 out of 29 BGCs. We propose therefore that MtrA is

308   a master regulator of secondary metabolism in *Streptomyces* species. Consistent with this,

309   unbiased metabolomics analysis of *S. venezuelae* shows that deleting *mtrB* results in a global

310   change in the metabolome. Given that secondary metabolite BGCs are often subject to

311   complex and multilevel regulation, and given the energy costs associated with making these

312   natural products, it is perhaps not surprising that we do not see obvious over-production of

313   other compounds in the Δ*mtrB* strains. We predict that deletion of known BGCs in the Δ*mtrB*

314   mutants and / or depletion of *mtrA* mRNA will have positive effects on the production of other

315   secondary metabolites. In summary, our study has revealed an important role for MtrA in the

316   life cycles of streptomycetes and our data suggest it might play a similar role to CtrA in

317   *Caulobacter crescentus*, acting as a master regulator of the cell cycle alongside other master

318   regulators such as BldD. Rapid progress has been made recently in elucidating the roles of

319   developmental regulators using *S. venezuelae* as a model system [16]. Our study shows that *S.*

320   *venezuelae* can also be used as a model to study global effects on secondary metabolism

321   and to elucidate the regulatory cascades that link secondary metabolism and differentiation.

322   Understanding these genetic circuits is essential if we are to unlock the full potential of these

323   bacteria.

324

325   **Materials and Methods**

326   **Strains, plasmids and primers.** The bacterial strains, plasmids and cosmids and primers

327   used in this study are listed in Tables S6-8. The Sv- and TB-MtrA expression vectors have

328   been deposited with AddGene (ID 85988-94). *S. venezuelae* NRRL B-65442 is deposited in

329   the USDA Agricultural Research Services (ARS) Culture Collection

330   (http://nrrl.ncaur.usda.gov/cgi-bin/usda/prokaryote/report.html?nrrlcodes=B%2d65442).

331   Plasmid stocks were prepared using Qiagen miniprep kits and cosmids were prepared as

332   described previously [37]. Genes were deleted using the ReDirect PCR targeting method [17] and

333   an *S. venezuelae* NRRL B-65442 cosmid library provided by Professor Mark Buttner at the

334   John Innes Centre, Norwich. All of the expression constructs used in this work were made by

335   Genscript by synthesising the *mtrA* alleles with or without 3' tags and then cloning into the

336   required vectors. Liquid cultures of *E. coli* were routinely grown shaking at 220 rpm in Lennox

337   Broth at 37°C. Liquid cultures of *S. coelicolor* or *S. venezuelae* were grown in Mannitol Yeast

338   Extract Malt Extract (MYM) at 30°C, shaking at 220 rpm. Cultures grown on solid MYM agar

339   were grown at 30°C. Spore stocks of *S. coelicolor* were prepared from cultures grown on Soya

340   Flour + Mannitol (SFM) agar. All media recipes have been published previously and

341   *Streptomyces* spores were prepared as described [37]. To determine the developmental growth

342   in liquid culture *S. venezuelae* and mutant strains were grown, shaking in 35ml MYM in 250ml

343   conical flasks containing springs at 30°C at 220rpm. A spore inoculum sufficient to reach an

344   OD_{600} of 0.35 after 8 hours of growth was added to 35ml of MYM media in 250 ml flasks

345   containing springs. The culture densities were measured at OD_{600}. Development in liquid

346   cultures was monitored using an GXML3000B microscope from GX optical. Pictures of agar

347   plate grown colonies were taken with a Zeiss SVII stereo microscope. SEM images were taken

348   at the bioimaging facility at the John Innes Centre.

349

350   **Purification of MtrA-His.** The *mtrA* gene was cloned into pETDuet1 (Novagene) to express

351   the protein with a C-terminal hexa-His tag and purified using a batch method. Cell pellets were

352   resuspended in 25ml lysis buffer (75mM Tris-HCl pH8, 20mM NaCl, 0.1% Triton X100, 50 µl

353   10mg/ml lysozyme, 3 x Pierce EDTA-free Protease Inhibitor Mini Tablets (Thermo Scientific)

354 in 1L) and incubated for 30 minutes at room temperature. The cell lysate was sonicated 2 x

355 40 seconds at 50Hz with 1 minute in between sonication steps. Cell debris was removed by

356 centrifugation at 18,000rpm for 20 minutes at 4°C in Beckman Coulter Avanti® J-20 high

357 performance centrifuge using a JLA-25-50 rotor (Beckman Coulter). The supernatant was

358 transferred in a fresh 50ml Falcon tube and 350µl of Ni-NTA agarose beads (Qiagen) were

359 added and incubated under gentle agitation for 1 hour at 4°C. The Ni-NTA agarose beads

360 were spun down gently (maximum of 1200rpm, 4°C) and the supernatant was discarded. The

361 Ni-NTA beads were resuspended in 2ml wash buffer (75mM Tris-HCl pH8, 200mM NaCl, 10%

362 glycerol, 10mM MgCl$_2$, 0.1mM DTT, 20mM 2-mercaptoethanol in 1L) and transferred in

363 Polypropylene columns (1ml, Qiagen). The beads in the column were washed with 20ml wash

364 buffer. The protein was eluted from the beads with 2.5ml elution buffer (wash buffer plus

365 350mL imidazole in 1L). Polyclonal antiserum was raised by Cambridge Research

366 Biochemicals. Immunoblotting was performed as described previously [38].

367

368 **DNA binding studies.** Chromatin Immunoprecipitation followed by sequencing (ChIP-seq)

369 was performed on *S. coelicolor* M145 Δ*mtrA* + MtrA-3xFlag and *S. venezuelae* NRRL B-65442

370   Δ*mtrA* + MtrA-3xFlag grown in liquid MYM medium [37]. Note that *S. coelicolor* M145 grows

371 vegetatively and does not differentiate under these growth conditions whereas *S. venezuelae*

372 NRRL B-65442 undergoes a full life cycle in 20 hours. Samples were taken at 16 and 20 hours

373 for *S. coelicolor* and at 8, 10, 12, 14, 16, 18 and 20 hours for *S. venezuelae*. ChIP-seq and

374 electrophoretic mobility shift assays (EMSAs) were performed as described previously [39] using

375 probes generated by PCR with 6-Fam labelled primers from Integrated DNA Technologies

376 (Table S8). ChIP-seq Gels were visualised using a Typhoon FLA 9500 laser scanner (GE

377 Healthcare) with LBP/BPB1 emission filter, Exmax 495nm Emmax 576nm, at 50µM resolution.

378 ChIP-seq data was analysed as described previously [40] and peaks were visually inspected

379 using integrated genome browser [41]. Binding sites were identified using MEME [19].

380

381   **RNA-sequencing.** Duplicate wild-type and Δ*mtrB* cultures were grown for 14 and 20 hours in

382   MYM medium and RNA was prepared as described previously [42]. Libraries were prepared and

383   sequenced by Vertis Biotechnologie and analysed using CLC Genomics Workbench.

384

385   **LCMS and PCA.**

386   Analytical HPLC was performed on an HPLC 1100 system (Agilent Technologies) connected

387   to a Gemini® 3 µm NX-C18 110 Å, 150×4.6 mm column (Phenomenex) and on a Synergi™

388   4 µm Fusion-RP 80 Å LC column 250×10 mm. UPLC-MS for metabolic profiling was performed

389   on a Nexera X2 liquid chromatograph (LC-30AD) LCMS system (Shimadzu) connected to an

390   autosampler (SIL-30AC), a Prominence column oven (CTO-20AC) and a Prominence photo

391   diode array detector (SPD-M20A). A Kinetex® 2.6 µm C18 100 Å, 100×2.1 mm column

392   (Phenomenex) was used. The UPLC-System was connected with a LCMS-IT-TOF Liquid

393   Chromatograph mass spectrometer (Shimadzu). UPLC-HRMS Data was acquired on an

394   Acquity UPLC system (Waters Corporation) equipped with an ACQUITY UPLC® BEH 1.7 µm

395   C18, 1.0 × 100 mm column (Waters Corporation) and connected to a Synapt G2-Si high

396   resolution mass spectrometer (Waters Corporation). A gradient between mobile phase A (H_2O

397   with 0.1 % formic acid) and mobile phase B (acetonitrile with 0.1 % formic acid) at a flow rate

398   of 80 µL/min was used. Initial conditions were 1 % B for 1 min, ramped to 90 % B within

399   6 minutes, ramped to 100 % B within 0.5 min, held for 0.5 min, returned to 1 % B within 0.1 min

400   and held for 1.9 min. MS spectra were acquired with a scan time of one second in the range

401   of m/z = 150 - 1200 in positive MSe-Resolution mode. The following instrument parameters

402   were used: capillary voltage of 3 kV, sampling Cone 40, source offset: 80, source temperature

403   of 120 °C, desolvation temperature 350 °C, desolvation gas flow 800 L/h. A solution of sodium

404   formate was used for calibration. A solution of leucine encephalin (H_2O/MeOH/formic acid:

405   49.95/49.95/0.1) was used as lock mass and was injected every 15 sec. The lock mass has

406   been acquired with a scan time of 0.3 sec and 3 scans were averaged each time. The lock

407   mass (m/z = 556.2766) has been applied during data acquisition.For processing

408   metabolomics data we used the Software Progenesis QI (Waters). All solvents for analytical

409 HPLC and UPLC-MS were obtained commercially at least in HPLC grade from Fisher

410 Scientific and were filtered prior to use. Formic acid (EZInfo 3.0 (MKS Umetrics AB) was used

411 for plotting PCA data.

412

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527

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537

Author contributions

539 FK and RFS constructed the *S. coelicolor* mutants and FK undertook phenotype analysis, NS

540 constructed all the *S. venezuelae* strains and constructs, analysed phenotypes and performed

541 ChIP-seq experiments, JTM performed RNA-seq and dRNA-seq experiments, JTM and GC

542 analysed the ChIP- and RNA-seq datasets, NH made constructs and prepared strain extracts

543 for LCMS, DH purified and analysed all natural products including LCMS and PCA analyses,

544 PAH, MIH and BW conceived the study and all the authors analysed data and wrote the

545 manuscript.

546