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Regulation of antibiotic production in Actinobacteria: new perspectives from the post-genomic era

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1 **Regulation of antibiotic production in Actinobacteria: new**
2 **perspectives from the post-genomic era**

3

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14 **Abstract**

15 The antimicrobial activity of many of their natural products has brought prominence to the
16 *Streptomycetaceae*, a family of Gram-positive bacteria that inhabit both soil and aquatic
17 sediments. In the natural environment, antimicrobial compounds are likely to limit the
18 growth of competitors, thereby offering a selective advantage to the producer, in particular
19 when nutrients become limited and the developmental programme leading to spores
20 commences. The study of the control of this secondary metabolism continues to offer
21 insights into its integration with a complex lifecycle that takes multiple cues from the
22 environment and primary metabolism. Such information can then be harnessed to devise
23 laboratory screening conditions to discover compounds with new or improved clinical
24 value. Here we provide an update of the review we published in NPR in 2011. Besides
25 providing the essential background, we focus on recent developments in our
26 understanding of the underlying regulatory networks, ecological triggers of natural product
27 biosynthesis, contributions from comparative genomics and approaches to awaken the
28 biosynthesis of otherwise silent or cryptic natural products. In addition, we highlight recent
29 discoveries on the control of antibiotic production in other Actinobacteria, which have
30 gained considerable attention since the start of the genomics revolution. New technologies
31 that have the potential to produce a step change in our understanding of the regulation of
32 secondary metabolism are also described.

33 1. INTRODUCTION

34 *Streptomyces* species are renowned for their ability to produce a multitude of bioactive
35 secondary metabolites, some of which have been co-opted clinically as a source of
36 antibacterial, anticancer, antifungal, antiparasitic and immunosuppressive agents¹⁻⁵. The
37 secondary metabolites produced by this taxon offer a chemical diversity that greatly
38 exceeds that of libraries of compounds synthesized chemically and have been pre-
39 selected through millions of years of evolution to interact effectively with biological targets.
40 With the development of numerous approaches for counter selecting compounds with
41 activities that have been previously characterised and in the case of antibiotics might have
42 been rendered ineffective by the emergence of resistance, natural products are being
43 revisited as a potential source of new pharmaceuticals^{6,7}.

44 The biological role of antibiotics has been a topic of some debate. Whilst antibiotics
45 in the natural habitat are typically regarded as weapons, in the same way as they are used
46 in the clinic⁸⁻¹⁰, it has been argued that at least some could function primarily in cell
47 communication and signalling¹¹⁻¹³. The latter view was based largely on the believe that
48 compounds with antibiotic activity are unlikely to reach concentrations in the soil that block
49 growth, as defined by the minimal inhibitory concentration (MIC). However, selection for
50 resistance occurs even at concentrations far below the MIC and antibiotic-sensitive strains
51 are demonstrably disadvantaged in competing for growth,¹⁴⁻¹⁶.

52 The majority of the antibiotics that are used in the clinic are produced by
53 actinobacteria, which are high G+C, Gram-positive bacteria. Of the actinobacteria,
54 perhaps the most prolific antibiotic producers are members of the genus *Streptomyces*,
55 which belong to the family *Streptomycetaceae*^{2, 17, 18}. Streptomycetes are found in
56 environments with varying nutrient supply, and metabolise a variety of carbon, nitrogen
57 and phosphate sources. To respond appropriately to the challenges imposed by the
58 environment, the genome of the model streptomycete *S. coelicolor* harbours a staggering

59 700 regulatory genes¹⁹. Streptomycetes have a multicellular life cycle, which culminates in
60 sporulation. The reader is referred elsewhere for details of this process²⁰⁻²⁴. In brief,
61 streptomycetes grow as non-motile, vegetative hyphae to produce a network of interwoven
62 filaments called vegetative mycelium. When reproduction is required, for example at the
63 time when nutrients run out, the vegetative mycelium acts as a substrate for newly formed
64 aerial hyphae that eventually differentiate into chains of unigenomic exospores.

65 Genes required for the transition from vegetative to aerial growth are typically
66 referred to as *bld* genes, referring to their bald phenotype, due to their failure to produce
67 the fluffy white aerial hyphae²⁵. Mutants that produce aerial hyphae but no spores are
68 referred to as *whi* mutants, for their white phenotype caused by the lack of the grey spore
69 pigment²⁶. Many of the *bld* and *whi* mutants that had been isolated in the 1970s by
70 phenotypic screening have later been identified by genetic complementation experiments,
71 and they have been instrumental in providing better insights into the regulatory cascades
72 that control morphological differentiation. For details we refer the reader to excellent
73 reviews elsewhere^{2, 23, 27-30}.

74 Production of bioactive compounds is typically linked to the developmental lifecycle,
75 and antibiotics are presumably produced to safeguard the nutrient supply during
76 developmental growth³¹⁻³³. Streptomycetes produce an arsenal of degradative enzymes
77 (e.g. glycosyl hydrolases, lipases and proteases), which combined with the production of
78 antibiotics and the ability to form desiccation-resistant exospores has facilitated their
79 success in a multitude of soil environments and sediments including those of marine and
80 freshwater ecosystems. The competitive attributes possessed by streptomycetes have not
81 gone unutilised by higher organisms. For instance, it has become clear that many insects,
82 animals and plants engage in protective symbioses with antibiotic-producing *Streptomyces*
83 species (reviewed in^{34, 35}). However, not all interactions between streptomycetes and
84 higher organisms are beneficial - a minority of species produce a cellulose synthase

85 inhibitor called thaxtomin and a coronafacic acid-like phytotoxin, which lead to the
86 development of scab diseases on potato and other tap-root crops ^{36, 37}.

87 Over the past 50 years, *S. coelicolor* has been the major model for the study of
88 antibiotic production and its control. Early on it was apparent that this strain produced
89 numerous natural products, including actinorhodin (Act; ³⁸), undecylprodigiosin (Red; ³⁹),
90 the calcium-dependent antibiotic (Cda; ⁴⁰) and plasmid-encoded methylenomycin (Mmy;
91 ⁴¹). The genes that encode the machinery for the production of these respective antibiotics
92 are clustered together in 'biosynthetic gene clusters' (BGCs), which typically also harbour
93 resistance gene(s) and one or more transcriptional regulators that control biosynthesis.
94 Sequencing of the *S. coelicolor* genome was a landmark event that revealed an
95 unexpected potential for the production of hitherto unidentified or cryptic natural products
96 ¹⁹, with more than 20 BGCs specifying a diverse range of secondary metabolites ^{42, 43}. One
97 of these is a so-called cryptic polyketide antibiotic (later named coelimycin), which is only
98 produced under specific growth conditions ^{44, 45}. Sequencing of other model actinobacteria
99 revealed a similar picture, with some species harbouring more than 50 different BGCs ⁴⁶⁻
100 ⁵¹. Thus, the potential of actinobacteria as producers of bioactive molecules was found to
101 be much greater than was initially thought. This prompted the sequencing and analysis of
102 the genomes of a large array of species to identify novel BGCs (reviewed in ⁵²⁻⁵⁵) plus the
103 development of approaches to induce the production of natural products under laboratory
104 conditions ^{1, 56-59}. The identification of BGCs is now relatively routine using bioinformatics
105 tools, such as antiSMASH ⁶⁰, CLUSEAN ⁶¹ and PRISM⁶². Available also are tools for the
106 identification of BGCs corresponding to specific classes of natural product, e.g.
107 NRPSPredictor for nonribosomal peptides ⁶³, BAGEL for bacteriocins and lantibiotics ⁶⁴
108 and SEARCHPKS for polyketides ⁶⁵. For a comprehensive overview of the available
109 bioinformatic tools for genome mining we refer the reader to excellent reviews elsewhere
110 ^{66, 67}.

111 This review is intended to be an update to our comprehensive review on the same
112 subject published in this journal in 2011³³. The broad subject is covered, but in the interest
113 of limiting duplicated content, the reader is often referred to our previous review. Here, the
114 focus lies on recent insights into the regulation of natural product biosynthesis in
115 streptomycetes, based on the literature from the period of 2011-2017. The article focuses
116 on both pleotropic and cluster-situated regulators, highlighting recent discoveries. We
117 thereby give specific attention to the control of antibiotic production in other actinobacteria.
118 We also provide an update on our understanding of the links between primary and
119 secondary metabolism and ecological triggers that stimulate natural product biosynthesis,
120 and outline methodology that could be used to activate silent or cryptic natural product
121 biosynthetic pathways.

122

123 **2. TRANSCRIPTIONAL REGULATION BY CLUSTER-SITUATED REGULATORS**

124 Over the last several decades, investigations into the regulation of the antibiotics produced
125 by *S. coelicolor* (Act, Red, Cda, Mmy and coelimycin) and that of streptomycin
126 biosynthesis by *S. griseus* have established key aspects of the regulation of secondary
127 metabolism in *Streptomyces*. For details we refer to reviews elsewhere³¹⁻³³. The
128 regulation of secondary metabolism is complex and frequently involves pleotropic global
129 regulators that either directly activate or repress biosynthetic genes or do so via cluster-
130 situated repressors or activators. A plethora of regulatory proteins is involved in the control
131 of antibiotic production, across a broad range of regulator families. and cross-regulation
132 results in a highly complex regulatory network. This is necessary to correctly interpret the
133 environmental signals and translate them into appropriate transcriptional responses, so as
134 to time the production of natural products, often closely connect to development. The
135 different families of transcriptional regulators known to be involved in the control of
136 antibiotic production, and some well-studied examples, are provided in Table 1.

137 The regulation of the BGCs for actinorhodin (Act; controlled by ActII-ORF4),
138 undecylprodigiosin (Red, controlled by RedD) and calcium-dependent antibiotic (Cda,
139 controlled by CdaR) of *S. coelicolor* and for streptomycin (Str, controlled by StrR) are the
140 most well-studied examples of cluster-situated regulators (CSRs). ActII-ORF4, CdaR and
141 RedD belong to the SARP family of *Streptomyces* antibiotic regulatory proteins⁶⁸, while
142 StrR unusually belongs to the family of ParB-Spo0J proteins, most of which are involved in
143 DNA segregation and sporulation⁶⁹. All available evidence supports the conclusion that
144 the cellular level of a cluster-situated regulator dictates the level of transcription of its
145 cognate BGC, which correlates closely with the level of production of the corresponding
146 natural product^{70, 71}. Indeed, the timing of Red production fully depends on the promoter
147 that drives the transcription of *redD*, allowing its use as a transcriptional reporter system⁷².
148 Thus, the ultimate factor deciding whether or not a BGC is expressed is its CSR(s). While
149 ActII-ORF4 and StrR act as single CSRs within their respective BGCs, production of RedD
150 is in turn controlled by RedZ^{73, 74}, which is related to the response regulators (RR) of
151 prokaryotic two-component systems (TCS) but 'orphaned', i.e. not genetically linked to a
152 histidine kinase⁷⁵. It is becoming increasingly clear that the presence of multiple CSRs is
153 more often the rule than the exception with each regulator effecting control of a subsets of
154 genes or contributing to a hierarchical cascade. The latter is exemplified by the BGCs
155 specifying polyene antifungal compounds such as amphotericin, nystatin, natamycin
156 (pimaricin) and candicidin⁷⁶⁻⁷⁹. It has been assumed and, in some cases, shown that
157 many regulators are responsive to small molecule signals. It has been assumed and in
158 some cases shown that many regulators are responsive to small molecule signals.
159 Regulators responsive to autoregulatory molecules such as γ -butyrolactones are well
160 known^{80, 81}, and feedback control by biosynthetic intermediates over production or export
161 has been demonstrated for jadomycin, Act and simocyclinone biosynthesis⁸²⁻⁸⁴. However,
162 the identity of the ligands/signals perceived by both pleotropic and CSRs is a major

163 question within the field, and if answered could lead to a revolution in chemical genetic
164 tools for the stimulation of natural product biosynthesis, and thus drug discovery.

165

166 **2.1. Pathway-specific regulation: streptomycin and actinorhodin as paradigms**

167 The first complete regulatory pathway leading to activation of a BCG was described for Str
168 in *S. griseus*⁸⁵. Transcription of StrR, which as mentioned above is the corresponding
169 CSR, is activated by the pleiotropic regulator AdpA (A-factor-dependent protein;⁸⁶, whose
170 transcription depends on the accumulation of the γ -butyrolactone 2-isocapryloyl-3R-
171 hydroxymethyl- γ -butyrolactone, better known as A-factor. The hormone-like compound
172 binds to ArpA⁸⁷, which acts as a repressor of *adpA* transcription⁸⁸. AdpA also activates
173 morphological differentiation, and thus plays a key role in the coordination of chemical and
174 morphological differentiation^{89, 90}. A-factor is synthesized by the enzyme AfsA⁹¹. The role
175 of A-factor in the control of antibiotic biosynthesis is further discussed in Section 9.

176 The transcription of *strR* is subject to multi-level control, and in particular by the
177 pleiotropic regulator AtrA^{92, 93}, which has an orthologue in *S. coelicolor* that activates
178 transcription of *actII-ORF4*, the CSR within the *act* cluster⁹⁴. Binding of AtrA *in vivo* within
179 the vicinity of the *actII-ORF4* promoter has recently been confirmed by chromatin
180 immunoprecipitation in combination with DNA sequencing (ChIP-seq) (McDowall *et al*,
181 unpubl. data). Compared to what is known about *strR*, the control of *actII-ORF4* is
182 complex with many transcription factors reported to control its expression directly.
183 Numerous direct and indirect regulators have been identified^{32, 33}. Some of the most
184 recent examples are summarized in Table 2. For some of these transcription factors,
185 binding has been demonstrated *in vivo* by ChIP-based approaches. In addition to AtrA,
186 these include DasR⁹⁵, a member of the GntR family that controls the uptake and
187 metabolism of N-acetylglucosamine (GlcNAc) and the degradation of chitin to GlcNAc⁹⁶,
188 ⁹⁷, AbsA2⁹⁸, the response regulator of the AbsA TCS, which negatively controls antibiotic

189 production in *S. coelicolor*^{99, 100}, AbrC3¹⁰¹, a response regulator of a TCS that is atypical
190 in having two histidine kinases¹⁰², and Crp¹⁰³, the cyclic AMP receptor protein, which is
191 perhaps best known for mediating carbon catabolite repression of the *lac* operon in *E. coli*
192¹⁰⁴, controls diverse cellular processes in many bacteria¹⁰⁵, and is a key regulator of
193 secondary metabolism as well as spore germination and colony development in *S.*
194 *coelicolor*¹⁰⁶. In addition to direct regulation, the expression of *actII-ORF4* is dependent on
195 *relA*¹⁰⁷, which is required for induction of the stringent response. The stringent response
196 enables bacteria to survive sustained periods of nutrient deprivation by enhancing the
197 transcription of numerous genes required to survive stress, while lessening transcription of
198 genes, such as those specifying stable RNAs, whose products are required in significantly
199 reduced amounts during periods of slowed growth^{108, 109}. Whilst the signals transduced by
200 Crp and the stringent response are well described, the signals sensed or transduced by
201 most of the transcription factors that bind the *actII-ORF4* promoter remain to be
202 elucidated. An exception is DasR, which is a receptor for glucosamine-6-phosphate (GlcN-
203 6P), an intermediate in GlcNAc metabolism, and derivatives⁹⁷. The binding of GlcN-6P by
204 DasR reduces its affinity for DNA, which de-represses the expression of genes that
205 facilitate the degradation of chitin to GlcNAc and its uptake and metabolism^{96, 97}. Links
206 between DasR and AtrA are described later in this review (Section 5.3).

207 In addition to AraC and AbsA, several other TCSs regulate secondary metabolism
208 in *S. coelicolor* and other actinobacteria¹¹⁰⁻¹¹³. TCSs are the major signal-transduction
209 systems of bacteria and enable them to monitor and adapt to environmental changes¹¹⁴,
210¹¹⁵. Streptomyces harbour a large number of TCSs, which likely reflects the changing
211 and variable nature of their natural habitats^{19, 110, 116}. The PhoRP TCS system is
212 ubiquitous in bacteria and senses phosphate and regulates its assimilation. PhoRP plays a
213 major role in the control of antibiotic production in streptomyces¹¹⁷⁻¹¹⁹. Similar has been
214 found for the AfsQ1/2 TCS, which controls the biosynthesis of Act, Red and Cda in

215 response to nitrogen limitation ¹¹¹ via what appears to be direct interaction with the
216 promoter regions of *actII-ORF4*, *redZ* (which activates *redD*) and *cdaR*, respectively. The
217 AfsQ1/2 TCS is closely related to CseBC, which responds to cell-envelope stress ⁷⁵.
218 Recently, it was shown that the DraRK TCS, which responds to high concentrations of
219 nitrogen ¹¹³, and the OsdRK TCS, which is oxygen-responsive, are similar in function to
220 the system controlling dormancy in mycobacteria ^{112, 120}, and are both required for Act
221 production. Interestingly, in the absence of a functional DraRK system the production of
222 Cpk and Red increases ¹¹³. The AbsA system has been exploited to improve the chance of
223 success during screening of streptomycetes for new antibiotics by overexpression of the
224 *S. coelicolor* homologue in other streptomycetes; this led among others to the induction of
225 pulvomycin production in *S. flavopersicus*. Cross-talk between the different regulatory
226 networks is discussed in Sections 5 and 6.

227

228 **2.2. Cross-regulation of disparate BGCs by cluster-situated regulators**

229 It is well established that a CSR usually binds to promoter sequence(s) and either
230 activates or represses genes only within its cognate BGC. For examples see Tables 1 and
231 2. However, this is not strictly true for all CSRs. Recently, the PAS-LuxR family cluster-
232 situated regulator within the candicidin BGC was shown to not only activate 16 out of the
233 21 genes in the gene cluster, but also to be required for expression of the antimycin BGC
234 ^{79, 121}. Thus, antimycin and candicidin biosynthesis are co-ordinately controlled by FscRI in
235 *S. albus* ¹²¹. A similar observation was made in *S. avermitilis*, where PteF, a member of
236 PAS-LuxR family and cluster-situated activator of the filipin BGC, was proposed to cross-
237 regulate the production of oligomycin ¹²². Thus, evidence is accumulating, at least for PAS-
238 LuxR family regulators, that they may not in fact simply be CSRs but act more broadly to
239 co-ordinately control the biosynthesis of multiple compounds. This is likely rooted in the
240 flexible inverted repeat the family of regulators appears to bind to both *in vitro* and *in vivo*

241 ^{121, 123}. It is an obvious and attractive hypothesis that production of secondary metabolites
242 with antimicrobial properties or subsets thereof should be coordinated, so as to maximise
243 any synergistic activity and minimise the development of resistance to the agents
244 produced.

245

246 **3. THE IMPACT OF PHOSPHATE AVAILABILITY ON SECONDARY METABOLISM**

247 The impact of phosphate availability on bacterial physiology and gene expression in
248 particular has been intensely studied in *Streptomyces* species and other bacteria ¹²⁴⁻¹²⁷.
249 Expression of a suite of genes involved in phosphate management termed the *pho* regulon
250 is controlled by the PhoRP TCS ^{116, 128, 129}. During phosphate starvation, the membrane-
251 bound sensor kinase, PhoR, undergoes autophosphorylation and transfers its phosphate
252 group to the response regulator, PhoP ^{119, 130} (Fig. 1). The phosphorylated form of PhoP
253 (PhoP-P) binds to a well conserved DNA motif called a PHO box and can either activate or
254 repress expression of genes within the *pho* regulon ¹¹⁸. During growth in phosphate
255 replete conditions, PhoR is prevented from phosphorylating PhoP via physical interaction
256 with the phosphate-specific transport (Pst) system, a high-affinity phosphate transport
257 system whose production is activated by PhoR ^{118, 131, 132}. This interaction creates a
258 regulatory loop in which the Pst system is produced at a low level during conditions of
259 phosphate sufficiency. When phosphate levels drop, PhoR is released and phosphorylates
260 PhoP, which then activates transcription of genes within the Pst system and the other
261 genes within the *pho* regulon ¹¹⁸. The precise signal that frees PhoR to phosphorylate
262 PhoP is unknown, but it is known that the switch is reversible.

263 It has been known for some 15 years that deletion of *phoP* can lead to earlier and
264 increased production of antibiotics ¹¹⁹. This phenomenon was covered in our previous
265 review ³³ and for *S. coelicolor* was rooted in destabilization of a negative regulatory loop
266 involving the AfsKRS system ^{133, 134}. AfsR is a transcription factor related to SARPs that

267 when phosphorylated by AfsK activates transcription of the gene encoding AfsS, a small
268 sigma factor-like protein required for antibiotic biosynthesis in *S. coelicolor*¹³⁵⁻¹³⁸. In the
269 proposed regulatory loop, PhoP represses the production of AfsS and AfsR represses the
270 production of PhoRP and the Pts system¹³⁵. However, recently PhoP was shown to in fact
271 be an activator of *afsS* transcription in experiments using a full panel of *phoP*, *afsR* and
272 *afsR/phoP* mutants and a suite of synthetic promoters engineered to prevent AfsR binding
273 but not PhoP binding¹³⁹. In a revised model, PhoP hinders higher activation of *afsS*
274 transcription by AfsR by outcompeting AfsR for binding to the *afsS* promoter (Fig. 1)¹³⁵,
275 ¹³⁹.

276 A series of ChIP-Chip experiments were conducted with *S. coelicolor*, which
277 provided genome-wide insight into the role of PhoPR in controlling secondary metabolism
278 ¹⁴⁰. These revealed that PhoP serves as a master regulator of secondary metabolism
279 during phosphate starvation, whereby it transiently represses pleiotropic activators of
280 antibiotic production and regulators of morphological development, namely *bldA*, which
281 specifies the leucine tRNA corresponding to the rare UUA codon, and *scbAR*, which
282 encodes the γ -butyrolactone regulatory system of *S. coelicolor* that positively influence
283 morphological development, and Act and Red biosynthesis^{141, 142}. Interestingly, the
284 ScbAR system also indirectly controls the gene expression of *scbR2* whose gene product
285 activates *afsK* expression¹⁴³, which is the cognate sensor kinase responsible for
286 activating the global regulator of secondary metabolism, AfsR (mentioned above). Thus,
287 although PhoP activates expression of *afsS*, it also indirectly represses transcription of
288 *afsK*, which means AfsR remains unphosphorylated and inactive (Fig. 1).

289 Although there are only a handful of example thus far, it is clear that in addition to
290 controlling pleiotropic regulators, PhoP can also act directly upon BGCs. For example, in *S.*
291 *coelicolor*, PhoP negatively regulates the biosynthesis of Cda by repressing the *cdaR*
292 gene¹⁴⁰. Interestingly, the inverse seems to be the case for the BGC specifying coelimycin

293 where there are three PHO boxes within the DNA sequence of two structural genes and
294 expression of the gene cluster appears to be PhoP-dependent ¹⁴⁰. Direct regulation of
295 biosynthetic pathways by PhoP is not a peculiarity of *S. coelicolor*, as PhoP was recently
296 shown to negatively regulate avermectin biosynthesis by repressing the expression of
297 *aveR*, which encodes a cluster-situated activator ¹⁴³.

298

299 **4. REGULATION OF SECONDARY METABOLISM BY NITROGEN**

300 The uptake and incorporation of nitrogen is essential for anabolism of amino acids, nucleic
301 acids and peptidoglycan, among other important macromolecules. *S. coelicolor* can utilise
302 diverse nitrogen sources including ammonia, nitrate, nitrite, urea, amino sugars and amino
303 acids ¹⁴⁴⁻¹⁴⁶. Assimilation of nitrogen results in the production of glutamate and glutamine,
304 which act as the primary nitrogen donors within the cell ¹⁴⁷. Like other bacteria,
305 *Streptomyces* species possess a sophisticated regulatory system that enables adaptation
306 to nitrogen availability. Many studies have indicated that the source of nitrogen can
307 influence the production of secondary metabolites. The production of most of the
308 secondary metabolites is reduced by nitrogen sources that are favourable for growth ¹⁴⁸,
309 ¹⁴⁹. This is presumably because utilization of a high-quality nitrogen source (e.g.
310 ammonium) causes more of the available carbon to be consumed for growth and
311 generation of biomass and thus ultimately less carbon is available for secondary
312 metabolism when starvation occurs. Although the above has been known for a long time,
313 the underpinning molecular detail has taken longer to elucidate. The global regulator
314 controlling nitrogen metabolism is GlnR, which is an orphan response regulator without a
315 cognate sensor kinase (Fig. 1) ^{145, 150}. Deletion of *glnR* in *S. coelicolor* blocks production of
316 Act and Red ¹⁵¹. GlnR-mediated regulation of Act and Red production was assumed to be
317 indirect until a recent study demonstrated otherwise. *In vitro* DNA binding and DNaseI
318 footprinting studies showed that GlnR binds the promoter sequence of CSRs within these

319 BGCs (*actII-ORF4* and *redZ*, respectively), implying that GlnR regulation is direct ¹⁵². In
320 the same study, direct regulation of CSRs of avermectin and oligomycin biosynthesis
321 (*aveR* and *olmRI/RII*, respectively) by GlnR in *S. avermitilis* was also demonstrated; thus,
322 direct regulation of a subset of natural product BGCs by GlnR is likely to be universal ¹⁵².
323 Several studies have recently been conducted that have enhanced the understanding of
324 nitrogen metabolism and its interconnectedness with phosphate and carbon utilization.
325 These connections and their implications for secondary metabolism are further discussed
326 in Section 6.

327

328 **5. CONTROL OF ANTIBIOTIC PRODUCTION BY THE CARBON SOURCE**

329 **5.1. Carbon catabolite repression and the control of antibiotic production**

330 In the natural environment, the availability of high-energy carbon sources, for instance,
331 glucose, promotes vegetative growth and suppresses morphological and chemical
332 differentiation ^{153, 154}. Examples of antibiotics whose production is repressed by glucose
333 include Act in *S. coelicolor* ^{155, 156}, chloramphenicol in *S. venezuelae* ¹⁵⁷, Str in *S. griseus*
334 ¹⁵⁸, and erythromycin in *Saccharopolyspora erythraea* ^{159, 160}. Like in most bacteria, carbon
335 utilization by streptomycetes is controlled by carbon catabolite repression (CCR), which
336 ensures that high-energy carbon sources such as glucose, fructose or TCA cycle
337 intermediates are utilized preferentially over energetically less favourable ones, such as
338 lactose, glycerol or mannitol. The best studied system is CCR by glucose, which is often
339 referred to as glucose repression ¹⁶¹⁻¹⁶⁴.

340 In most bacteria, glucose is transported through the phosphoenolpyruvate-
341 dependent phosphotransferase system or PTS. The PTS encompasses Enzyme I (EI) and
342 phosphocarrier protein HPr in combination with carbohydrate-specific transport complexes
343 called Enzyme II (EII), which confer substrate specificity ^{165, 166}. As a result, the PTS

344 typically plays a key role in glucose repression ^{104, 167, 168}. However, in *Streptomyces*
345 species, deletion of either of the genes *ptsH*, *ptsI* or *crr* for HPr, EI and EIIA, respectively,
346 has no influence on CCR, but instead leads to a block in morphological differentiation, with
347 mutants failing to produce aerial hyphae and/or spores on a reference medium such as
348 R2YE agar ^{97, 169}. This sporulation defect is surprising and may be associated with lack of
349 iron and/or copper in this medium, accompanied by a reduced production of the
350 siderophore, desferrioxamine ¹⁷⁰⁻¹⁷². This link between carbon availability, iron
351 homeostasis and morphological differentiation has not yet been resolved. The limited role
352 of the PTS in CCR may be explained by the fact that in streptomycetes, glucose is
353 internalized via the GlcP permease, which belongs to the major facilitator subfamily of
354 transporters ¹⁷³⁻¹⁷⁵. For a summary of central carbon metabolism and CCR, see Fig. 2.

355 It was recognized many decades ago that randomly generated mutants lacking
356 CCR are invariably mutated in the gene *glkA*, which encodes a glucose kinase ^{176, 177}.
357 Indeed, a targeted deletion of *glkA* in a clean genetic background was pleiotropically
358 defective for CCR ¹⁷⁸⁻¹⁸⁰. The activity of Glk is mediated by as of yet unknown mechanism
359 ¹⁸¹. Its role in catabolite repression may be co-ordinately controlled with a number of other
360 proteins. These include SCO2127, a protein of unknown function, which is encoded by the
361 gene upstream of *glkA* ^{182, 183} and regulatory proteins that control the transcriptional
362 network of genes that mediate CCR, such as the global regulators Rok7B7 and DasR (see
363 below). Another interesting protein is the phosphoinositide phosphatase, SblA ¹⁸⁴. Deletion
364 of *sblA* in *Streptomyces lividans* leads to relief of CCR, with accelerated growth and
365 development in the presence of glucose on some media ¹⁸⁵. These phenotypes correlated
366 with reduced glucose uptake by the mutant and may therefore affect the activity of GlcP.
367 The cleavage of phosphoinositides by SblA is apparently required to resume growth in
368 transition phase, although the mechanism has not been elucidated ¹⁸⁵.

369 Studies with *S. peucetius* suggested the existence of an integral regulatory system
370 that responds to glucose transport and metabolism, which probably elicits CCR¹⁵⁴.
371 Indeed, addition to growth media of either of the glycolytic intermediates fructose 1,6-
372 biphosphate and phosphoenolpyruvate results in glucose repression of daunorubicin and
373 doxorubicin biosynthesis in *S. peucetius*¹⁸⁶. This connects to observations that the activity
374 of GlkA depends on interaction with the glucose permease GlcP in *S. coelicolor*¹⁸¹.

375 Many antibiotics show growth phase-dependent control. As a consequence,
376 developmental mutants that are blocked in an early phase of the life cycle - in particular
377 *bld* mutants - typically fail to produce antibiotics. A well-studied case is represented by
378 mutants that lack the developmental gene, *bldB*, as these are not only disturbed in
379 development and antibiotic production, but are also defective in CCR^{187, 188}. This links the
380 pathways that regulate carbon utilization and morphological differentiation. BldB is a
381 member of a family of DNA-binding proteins that are only found in Actinobacteria. The
382 family is widespread in streptomycetes, with several paralogues in *S. coelicolor*, including
383 AbaA and WhiJ, which play a role in the control of antibiotic synthesis and development,
384 respectively¹⁸⁹. Identification of the BldB regulon and the way its activity is modulated will
385 likely offer important new insights into the growth phase-dependent control of antibiotic
386 production and the role of CCR in this process.

387

388 **5.2. New insights into the nutrient-sensory DasR system**

389 In streptomycetes, the PTS plays a major role as the first step in a global antibiotic
390 sensory system revolving around the nutrient sensory protein, DasR, which is conserved
391 in streptomycetes and many other actinobacteria. DasR is a GntR-family repressor with a
392 pleiotropic role in the regulation of primary and secondary metabolism and of
393 development. For details, we refer to reviews elsewhere^{33, 190}. Here we summarise the
394 key elements of the regulon and highlight recent insights (Fig. 3). The core regulon of

395 DasR in all Gram-positive bacteria revolves around the genes for aminosugar transport
396 (*pts*) and metabolism (*nag*) and in streptomycetes also the genes for the chitinolytic
397 system (*chi*). Originally identified as the repressor of the chitobiose transporter DasABC
398 ^{191, 192}, it was soon recognized that DasR also controls many genes involved in antibiotic
399 production. Comprehensive analysis of the DasR regulon of *S. coelicolor* showed that it
400 acts as a direct and very global transcriptional repressor of antibiotic production by binding
401 to the promoter regions of the CSRs for all known chromosomally located antibiotic BCGs
402 in *S. coelicolor* ^{95, 97, 193, 194}. DasR also represses siderophore biosynthesis via control of
403 the iron-homeostasis regulator *dmdR1* ^{170, 195}. A similar pleiotropic role of DasR has also
404 been reported in the erythromycin producer *S. erythraea* ^{196, 197}, but is not typical of all
405 streptomycetes.

406 The DNA-binding activity of DasR is modulated by ligands derived from GlcNAc or
407 glucosamine (GlcN), in particular GlcNAc-6P and GlcN-6P, and the crystal structure of
408 DasR and its orthologue NagR of *Bacillus subtilis* in complex with these ligands have been
409 elucidated ^{198, 199}. GlcN-6P stands at the cross-roads of carbon and nitrogen metabolism
410 and cell-wall synthesis, and by acting as an effector of the DasR-dependent antibiotic
411 control system, it plays a major role in the connection between primary and secondary
412 metabolism (Fig. 3). The DNA-binding activity of DasR depends on environmental
413 conditions. High concentrations of GlcNAc under famine conditions (e.g. on minimal
414 media) result in inactivation of DasR, and thus derepression of its targets, leading to
415 enhanced antibiotic production and development. Conversely, on rich media, GlcNAc
416 represses antibiotic and development, leading to a complete developmental block ^{97, 194,}
417 ²⁰⁰. This phenomenon is known as *feast* or *famine*; under conditions of nutritional richness,
418 aminosugars are perceived as derived from chitin, signalling plenty of nutrients, while
419 under poor growth conditions (famine) it is perceived as coming from autolytic degradation
420 of the cell wall and hence cell death. The latter elicits development and antibiotic

421 production. Besides the phosphorylated aminosugars GlcN-6P and GlcNAc-6P, other
422 metabolites may also modulate the DNA-binding activity of DasR. These include high
423 concentrations of phosphate (organic or inorganic), which were shown to enhance the
424 binding of DasR to its recognition sites^{95, 201}. Thus, the affinity of DasR for its recognition
425 sites (and with that the expression of its regulon, including many BGCs for natural
426 products) depends on the metabolic status of the cell. Interestingly, high concentrations of
427 phosphate (either organic or inorganic) enhance binding of DasR to its recognition site *in*
428 *vitro*, which reinforces the PhoP-mediated repression of antibiotic production by phosphate
429^{95, 201}.

430 Full genome-scale identification of the DasR binding sites *in vivo* using ChIP-chip
431 analysis corroborated the identity of canonical DasR binding sites or *dre* (DasR-responsive
432 elements), but also revealed so-called class II sites, which do not conform to the known
433 consensus sequence⁹⁵. These sites are not found by the regulon prediction algorithm
434 PREDetector²⁰². Binding of DasR to class II sites may require a co-repressor, which has
435 not yet been identified. The ChIP-Chip analysis also showed that the binding profile of
436 DasR changes dramatically over time, with only small overlap in the binding profiles
437 between 24 (vegetative growth) and 54 hours (morphological differentiation and antibiotic
438 production). Thus, the DasR regulon is a highly complex system, which is influenced by
439 metabolic status and most likely also by other regulatory proteins. Taken together, the
440 metabolic status of the cell determines the selectivity of DasR for its recognition sites and
441 thus the expression of its regulon, which includes many secondary metabolite BGCs.

442

443 5.3. Competition between AtrA, Rok7B7 and DasR and connections to CCR

444 Until the discovery of DasR, it was unclear how global carbon control was related to the
445 control of specific carbon utilization regulons and antibiotic biosynthetic genes. Deletion of
446 the genes for either GylR or MalR relieves both CCR and substrate induction of glycerol

447 and maltose utilization, respectively, and hence gives constitutive expression even in the
448 absence of inducer, while over-expression results in hyperrepression^{203, 204}. This suggests
449 that a global regulatory system for carbon utilization does not exist in *S. coelicolor*. In most
450 bacteria, global carbon control depends on the cAMP receptor protein (CRP).
451 Streptomycetes do have a cAMP receptor protein, but in contrast to other bacteria, it does
452 not seem to play a role in CCR. Instead, CRP plays a role in the control of germination,
453 and *crp* null mutants show prolonged dormancy¹⁰⁶. Importantly, genome-wide DNA
454 binding studies and transcriptional analysis revealed that CRP also globally controls
455 antibiotic BGCs in *S. coelicolor* (¹⁰³; see also section 6).

456 There is also growing evidence that besides DasR, the TetR-family regulator AtrA
457 plays a role in carbon utilization (Fig. 4). Very recent ChIP-seq experiments (McDowall *et*
458 *al.*, unpubl. data) have confirmed that AtrA binds upstream of *nagE2*, which encodes a
459 known permease for the uptake of GlcNAc²⁰⁵. Similar to what was found for *actII-ORF4*,
460 this binding appears to activate transcription as disruption of *atrA* results in reduced levels
461 of *nagE2* transcript (Nothaft *et al.*, 2010). This led to the suggestion that AtrA may increase
462 Act production indirectly through enhanced GlcNAc-induced inactivation of DasR as well
463 as directly through activation of *actII-ORF4* transcription (Nothaft *et al.*, 2010). The control
464 of DasR activity by AtrA via cellular levels of GlcNAc may extend beyond *nagE2* as recent
465 ChIP-seq also identified AtrA binding to recognisable motifs upstream of SCO0481, which
466 encodes a protein that binds chitin (a rich source of GlcNAc), and *crr* (SCO1390), for the
467 global PTS component EIIA, that is required for GlcNAc transport. The role of AtrA in
468 carbon utilisation almost certainly extends beyond GlcNAc metabolism (Fig. 4). ChIP-seq
469 also identified AtrA binding to sites upstream of *gylR* (SCO1658) and *glpk2* (SCO0509),
470 which encodes a glycerol kinase outside the *gyl* operon. Control of morphological
471 differentiation by AtrA is explained at least in part by transcriptional control of *ssgR* (Fig. 4)
472 ²⁰⁶, the transcriptional activator of the gene encoding SsgA, which is involved in cell

473 division and sporulation^{207, 208}. Disruption of *atrA* suggests it activates transcription of
474 *ssgR*²⁰⁶, and direct binding of AtrA within the upstream regulatory region of *ssgR* was
475 confirmed by ChIP-seq (McDowall et al, unpubl. data).

476 The ROK-family protein, Rok7B7 takes up an interesting position in the regulatory
477 network as it connects the control of antibiotic production and carbon catabolite repression
478²⁰⁹. Mutants lacking *rok7B7* are delayed in their developmental programme and are
479 pleiotropically disturbed in terms of antibiotic production, perhaps as a consequence of a
480 yet unexplained change in CCR. Rok7B7 activates the transcription of *actII-ORF4* (and
481 hence Act production) and represses the biosynthesis of Red and Cda, although its
482 binding site has so far not been identified^{209, 210}. Aside from *actII-ORF4*, Rok7B7 also
483 activates the GlcNAc *pts* gene, *nagE2*, which means it counteracts the activity of DasR in
484 a manner very similar to AtrA.

485 The signals that are required for activation of AtrA and Rok7B7 are unknown. Since
486 AtrA is a TetR-regulator it is suggested that this protein is regulated in an allosteric manner
487 by a ligand to exert its effect on secondary metabolism. In *S. globisporus*, AtrA is inhibited
488 by the binding of heptaene, a biosynthetic intermediate of lidamycin whose biosynthesis is
489 controlled by AtrA via activation of its CSR²¹¹. As part of this work, it was also reported
490 that the DNA-binding activity of *S. coelicolor* AtrA is regulated by Act²¹¹. Whilst this finding
491 was shown with different preparations of Act, the specificity of this effect needs to be
492 evaluated further. To our knowledge, in all streptomycetes *atrA* is co-located with a
493 divergent AtrA-target gene (SCO4119 in *S. coelicolor*) that encodes NADH
494 dehydrogenase²¹². There is interest in identifying the substrate of SCO4119 as at least
495 some members of the TetR family interact with ligands that are structurally identical or
496 related to the substrates of proteins encoded by genes divergent to their own²¹³. As ChIP-
497 chip experiments failed to show binding of ROK7B7 to genomic DNA under standard
498 growth conditions on minimal media, it was proposed that the regulator requires a co-

499 factor or ligand to facilitate its DNA binding activity. The control of - and gene synteny with
500 - the xylose transport operon *xyIEFG* by Rok7B7 hints at C5-sugars as candidate ligands
501 for this regulator²⁰⁹.

502 Interestingly, there is an intricate link between Rok7B7, DasR and CCR, which in
503 turn has important implications for the control of antibiotic production. Proteomic
504 comparison of *S. coelicolor* and a *glkA* null mutant showed that glucose activates the
505 expression of Rok7B7 in a Glk-independent manner ²¹⁴, which was later confirmed by
506 transcriptomic analysis ²¹⁵. In turn, DasR and Rok7B7 repress the expression of *glkA* and
507 thus CCR ^{95, 209}, while conversely, Glk represses Rok7B7 ²¹⁴. Deletion of *rok7B7* results in
508 a loss of CCR, which directly implicates Rok7B7 in CCR ^{214, 215}. It is unlikely however that
509 *glkA* is a member of the *rok7B7* regulon, as *glkA* transcription is constitutive, and its
510 activity is post-translationally controlled ^{181, 215}.

511 In summary for this chapter, there are multiple regulatory networks that
512 connect carbon control to the control of antibiotic production. Understanding carbon
513 source-dependent control of antibiotic production is important from the perspective of both
514 the design of growth media for yield optimization and for screening of new bioactive
515 molecules. Despite the wealth of literature, it is still unclear how Glk exerts CCR, and we
516 expect that more regulatory proteins that play a role in this important process will be
517 discovered. It is becoming clear that there is a strong connection to the regulons of DasR,
518 Rok7B7 and AtrA. Future research will need to elucidate precisely how this multi-layer
519 control network is governed. Finding the ligands for AtrA and Rok7B7 would be one of the
520 major steps to take.

521

522 **6. CONNECTIONS BETWEEN PHOSPHATE, NITROGEN AND CARBON**
523 **METABOLISM**

524 Carbon, nitrogen and phosphate are essential components for the basic building blocks of
525 all cellular life. It is reasonable to assume that acquisition and utilization of these elements
526 would be coordinately controlled. Although widely accepted, molecular characterization of
527 this interconnectivity has only emerged recently, with the important discovery that GlnR,
528 DasR and CRP jointly regulate three genes for citrate synthesis in the erythromycin
529 producer *S. erythraea*²¹⁶. CRP controls early processes during growth in *Streptomyces*
530 species^{106, 217} and acts as a global regulator of Act, Cda and Red production, perhaps by
531 coordinating precursor flux¹⁰³. Indeed, 8 out of 22 secondary metabolic clusters on within
532 the *S. coelicolor* genome harbour Crp binding sites, suggesting a pleiotropic role in control
533 of antibiotic production. Further evidence for the connection between C- and N-metabolism
534 via GlnR came from elegant experiments showing that several ABC transporter systems
535 are under direct control of GlnR in *S. erythraea*, affecting growth on maltose, mannitol,
536 mannose, sorbitol and trehalose²¹⁸. Recent data show that in *S. coelicolor*, GlnR is
537 activated by glucose²¹⁵, while GlnR directly activates transcription of a putative
538 carbohydrate transport operon *agl3EFG*²¹⁹. Taken together, these data suggest direct
539 linkage between carbon and nitrogen metabolism, albeit perhaps only when certain carbon
540 sources are available.

541 The understanding of links between nitrogen and phosphate metabolism in *S.*
542 *coelicolor* is better developed. PhoP and GlnR control antibiotic production in response to
543 the availability of phosphate and nitrogen sources, respectively^{135, 220}. Similar to the
544 competitive activation of *afsS* by AfsR and PhoP described in section 3, these two
545 regulators bind to overlapping regions within the *glnR* promoter, but unlike the *afsS* story,
546 PhoP represses *glnR* transcription while only AfsR promotes it¹³⁹ (Fig. 1). When
547 phosphate is plentiful, PhoP is inactive and thus AfsR (dependent on the growth phase)
548 activates transcription of *glnR*, but when phosphate is in short supply, PhoP is
549 phosphorylated by PhoR and represses the expression of *glnR* (Fig. 1)²²⁰. In addition,

550 PhoP also directly represses transcription of genes within the GlnR regulon, namely two
551 glutamine synthetases (*glnA* and *glnII*) and the promoter for the *amtB-glnK-glnD* operon,
552 which encodes an ammonium transporter and putative nitrogen sensing/regulatory
553 proteins ²²¹. Uptake/utilization of nitrogen is presumably superfluous if insufficient
554 phosphate is available, hence the PhoP-mediated repression of genes involved in these
555 processes. Thus, PhoP-mediated control of nitrogen metabolism may help balancing the
556 cellular P/N equilibrium.

557 Connection between phosphate and carbon metabolism is less well studied, but
558 one link may be governed via the PhoP-controlled enzyme PPK (polyphosphate kinase),
559 which affects antibiotic production in response to the level of inorganic phosphate (Pi) ¹²⁷,
560 ²²². PPK is involved in maintaining the cellular energy balance by regenerating ATP from
561 ADP and polyphosphates and *ppk* mutants show enhanced Act production under Pi-
562 limited growth conditions ¹²⁷. This was recently explained by increased degradation of
563 triacylglycerols (TAGs), resulting in accumulation of the polyketide precursor acetyl-CoA
564 ²²³. Additionally, phospho-sugars inhibit antibiotic production in streptomycetes. This effect
565 is mediated by the phosphate- rather than of the glyco-moiety, as the inactivation of *phoP*
566 or *ppk* prevents or enhances, respectively, their utilization as nutrient sources and their
567 inhibitory effect on antibiotic production²²⁴.

568 Thus, it is becoming evident that the conventional understanding of the PhoRP,
569 AfsR and GlnR as the elements of the linear transduction systems regulating primary and
570 secondary metabolism have been revised significantly over the last several years. Recent
571 discoveries made it possible to understand, at least partially, the cross-talk occurring
572 between regulators for phosphate and nitrogen metabolism, and to a lesser extent carbon
573 metabolism in streptomycetes. It is a reasonable expectation to predict that established
574 methods for assessing DNA binding *in vivo* (i.e. ChIP-seq ²²⁵ in combination with new

575 strategies for robustly mutagenizing and identifying mutants (i.e. Tn-Seq²²⁶ will enhance
576 the ability to probe these regulons and their cross regulation.

577

578 **7. THE IMPACT OF METALS ON SECONDARY METABOLISM**

579 Iron is an essential metal that plays important roles in DNA replication, protein synthesis
580 and respiration. Iron is relatively unavailable in the soil due to the low solubility of the Fe³⁺
581 ion under aerobic conditions at neutral pH. Production of iron-chelating compounds called
582 siderophores is the most common way that bacteria circumvent this problem²²⁷.
583 Moreover, some bacteria have developed systems that allow them to utilize siderophores
584 synthesised by neighbouring microorganisms^{171, 228, 229}. The primary impact of iron
585 deficiency in *Streptomyces* and other bacteria, is the stimulation of siderophore
586 production. All *Streptomyces* species examined thus far appear to harbour a BGC for
587 desferrioxamine, which has been proposed to be part of the 'core' secondary metabolome
588 of the genus²³⁰, while other streptomycetes produce additional siderophores; *S. coelicolor*
589 and *S. scabies* produce coelichelin and pyochelin, for example^{231, 232}. Production of
590 desferrioxamine is normally repressed by the DmdR1 protein, which becomes
591 derepressed in the absence of iron²³³⁻²³⁵. The *dmdR1* gene is unusual in that its DNA
592 sequence encodes a second gene (*adm*) using the anti-sense strand of DNA²³⁶. Deletion
593 of the *dmdR1-adm* locus in *S. coelicolor* abolished sporulation and the production of Act
594 and Red²³³. Subsequent experimentation whereby either *dmdR1* or *adm* were individually
595 mutated by a point mutation revealed that inactivation of *dmdR1* had no impact on Act and
596 Red production where as these compounds were overproduced when only *adm* was
597 mutated²³⁶. Another link between iron availability and secondary metabolism in *S.*
598 *coelicolor* is that iron de-represses the pleiotropic TCS, AbrA1/A2, which negatively
599 regulates Act and Red production, although the mechanism has not yet been resolved²³⁷.

600 Zinc is an important transition metal required as a cofactor for many enzymes and
601 regulatory proteins important for normal bacteria physiology. However, the intracellular
602 free level of this element should be maintained within a narrow range due to its potential
603 toxicity^{238, 239}. Its uptake in streptomycetes as well as in other bacteria is regulated by Zur,
604 a zinc-responsive transcriptional regulator^{240, 241}. Interestingly, there is a Zur-binding site
605 within the BGC for the metal chelator, coelibactin and adjacent to this is a binding site for
606 another zinc-sensitive regulator, AbsC; together these regulators repress coelibactin
607 biosynthesis²⁴². Interestingly, AbsC also seems to be required for the production of Act
608 and Red when *S. coelicolor* is cultivated under the specific conditions of zinc limitation and
609 inactivation of *zur* and *absC* genes block sporulation. Binding of AtrA upstream of the
610 promoter for *zur*²⁴³ has been identified both biochemically and by ChIP-seq (McDowall et
611 al, unpubl. data) suggesting yet another layer of regulation that potentially facilitates
612 integration with primary metabolism as well as secondary metabolism and morphological
613 development. More detailed study of these regulators is necessary in order to fully
614 illuminate their regulons and the nature in which they overlap and interconnect with other
615 metal acquisition systems. *Amycolatopsis japonicum* produces the biodegradable
616 ethylenediamine-tetra acetate (EDTA) isomer [S,S]-EDDS, whose gene cluster was
617 elucidated²⁴⁴. Trace amounts of zinc in the culture media inhibit the production of [S,S]-
618 EDDS, which led to the proposal that the molecule is required for zinc uptake. The
619 synthesis of the zincophore is repressed by the zinc regulator Zur²⁴⁴.

620 Recently, the impact of rare earth elements (REEs) on secondary metabolism was
621 explored. Supplementation of culture medium with scandium or lanthanum stimulated the
622 production of Act by *S. coelicolor*, Str by *S. griseus* and actinomycin by *S. antibioticus*²⁴⁵.
623 Although precise mechanistic detail is lacking, scandium stimulation of Act production is
624 dependent on the ppGpp synthetase, RelA and is mediated by upregulation of actII-ORF4
625²⁴⁵. Interestingly, scandium was also able to rescue the ability of *S. lividans* to produce Act,

626 a compound that the species does not normally produce despite harbouring a nearly
627 identical gene cluster ²⁴⁵. Quantitative RT-PCR and HPLC analyses showed that in
628 addition to Act, scandium supplementation stimulated the expression of eight other BGCs
629 in *S. coelicolor* ²⁴⁶. Stimulation of secondary metabolism by REEs is not restricted to
630 actinobacteria – scandium was recently shown to elicit the production of amylase and
631 bacilysin in *B. subtilis* ²⁴⁷. Thus, REEs represent a relatively unexplored method for
632 activating the expression of silent or weakly expressed BGCs and future studies should be
633 aimed at understanding the molecular mechanism(s) by which this occurs.

634

635 **8. MORPHOLOGICAL DEVELOPMENTAL CONTROL OF ANTIBIOTIC PRODUCTION**

636 As mentioned in the introduction to this review, the production of antibiotics (and other
637 secondary metabolites) is temporally correlated to the onset of development of
638 *Streptomyces* colonies ^{31, 33}. A model of the linkage between the control of antibiotic
639 production and development is presented in Fig. 5. A likely explanation is that the colony is
640 particularly vulnerable to competitors when it is undergoing programmed cell death (PCD),
641 and antibiotics are produced to protect the colony and the nutrients released during PCD.
642 Until recently, the occurrence of PCD in bacteria has been a subject to major debate, but it
643 is becoming increasingly clear that PCD plays a major role the life cycle of multicellular
644 bacteria ^{22, 248-250}, and in that of streptomycetes in particular ^{251, 252}. A direct link between
645 PCD and antibiotic production was demonstrated with the discovery that GlcNAc, which
646 together with N-acetylmuramic acid forms the peptidoglycan strands, acts as an elicitor of
647 antibiotic production via metabolic inactivation of the global antibiotic repressor DasR ^{194,}
648 ²⁵³. For details we refer to section 5. Interestingly, production of prodiginines, which have
649 anticancer activity by degrading the DNA, may play a direct role in triggering PCD in *S.*
650 *coelicolor*, and mutants that fail to produce prodiginines have strongly reduced PCD,
651 whereby vegetative growth is prolonged ²⁵⁴.

652 As a consequence of the growth phase-dependent control of antibiotic production,
653 developmental mutants that are blocked in an early phase of the life cycle - in particular
654 *bld* mutants - typically fail to produce antibiotics. As mentioned in Section 5.1, mutants of
655 the developmental gene *bldB* are not only disturbed in development and antibiotic
656 production, but are also defective in CCR^{187, 188}. This links the pathways that regulate
657 carbon utilization and morphological differentiation. BldB is a member of a family of DNA-
658 binding proteins that are only found in Actinobacteria. The family is widespread in
659 streptomycetes, with several paralogues in *S. coelicolor*, including AbaA and WhiJ, which
660 play a role in the control of antibiotic synthesis and development, respectively¹⁸⁹.
661 Identification of the BldB regulon and the way its activity is modulated will likely offer
662 important new insights into the growth phase-dependent control of antibiotic production
663 and the role of CCR in this process.

664 BldD is a small DNA-binding protein that is required for development and antibiotic
665 production (Fig. 5)²⁵⁵. BldD is related to SinR, a master regulator of the transition from the
666 motile to a sessile state in *Bacillus subtilis*, and hence associated with the control of biofilm
667 formation^{256, 257}. The BldD regulon encompasses over 150 transcriptional units, many of
668 which are involved in the control of development²⁵⁸. One of its targets is *bldA*, which at
669 least in part explains the requirement of BldD for antibiotic production. BldD binds to DNA
670 as a homodimer, and dimerization is dependent on the binding of a tetramer of the
671 signalling molecule cyclic-di-GMP²⁵⁹. This is another interesting example of small
672 molecule-based control of antibiotic production in *Streptomyces*.

673 Other *bld* mutants also fail to produce antibiotics, but the phenotype of these
674 mutants is not independent of the growth medium (Fig. 5). In fact, *bldA*, *bldC*, *bldG*, *bldH*
675 (*adpA*), *bldJ* and *bldK* mutants produce spores on non-repressing carbon sources such as
676 mannitol or glycerol, but not on media containing glucose. Interestingly, mutation of *glkA*
677 restores antibiotic production and morphological development to *bldA* mutants³³, while

678 *bldJ* and *bldK* mutants are rescued by supplementing the colonies with iron. The latter is
679 due to their failure to produce the siderophore desferrioxamine¹⁷⁰. In fact, most *bld*
680 mutants are affected in desferrioxamine biosynthesis, with strongly reduced production of
681 the siderophore in *bldA*, *bldJ*, and *ptsH* mutants, and overproduction in *bldF*, *bldK*, *crr* and
682 *ptsI* mutants¹⁷⁰.

683 An infamous example of translational control of development and antibiotic
684 production is BldA, the tRNA that recognizes the rare UUA codon for leucine. Mutants of
685 *S. coelicolor* defective in *bldA* have a bald phenotype and fail to produce antibiotics^{260, 261}.
686 The latter is a direct consequence of the presence of UUA codons in the mRNA of the
687 genes for ActII-ORF4 and RedZ^{73, 74}. The presence of TTA codons in BGCs for
688 specialized metabolites - and in particular in genes encoding CSRs - is more a rule than
689 an exception, which provides strong phylogenetic evidence for the fact that control of
690 antibiotic production by BldA has evolved with a purpose²⁶².

691 Mutants that are blocked in sporulation (so-called *whi* mutants) generally are not
692 affected in antibiotic production. This is most likely because the decisions to switch on
693 secondary metabolism made at an earlier stage in the life cycle. The exception is *ssgA*,
694 whose transcription does not depend on any of the 'classical' *whi* genes²⁰⁷. SsgA
695 activates sporulation-specific cell division by controlling the localization of its paralogue
696 SsgB, which in turn recruits FtsZ to initiate sporulation-specific cell division (Fig. 5)²⁶³. In
697 contrast to most developmental control proteins, SsgA and SsgB lack DNA-binding
698 domains. The SsgA-like proteins are unique to sporulating actinobacteria, and most likely
699 function as chaperones that recruit multi-component complexes^{264, 265}. Over-expression of
700 *ssgA* results in overproduction of prodiginines (Red), while Act production is blocked²⁶⁶,
701²⁶⁷. The most likely explanation is that SsgA blocks *S. coelicolor* development at a stage
702 corresponding to early aerial growth, where Red production has been switched on, while
703 Act production has not yet been initiated. SsgA and SsgB probably represent another

704 important link in the coordination of secondary metabolite production with vegetative
705 growth ²⁶⁸.

706 WblA is a member of the WhiB-like proteins, and 11 paralogues are encoded by the
707 *S. coelicolor* chromosome ²⁶⁹. The Wbl proteins are small iron-sulphur proteins that are
708 unique to actinobacteria. Disruption of *wblA* has a highly pleiotropic effect on overall gene
709 expression in *S. coelicolor* and prevents development while strongly increasing antibiotic
710 production in this organism ²⁶⁹. Conversely, overproduction of WblA pleiotropically
711 represses the biosynthesis of Act, Red and Cda in *S. coelicolor* and of anthracyclines in *S.*
712 *peucetius* ²⁷⁰. Deleting *wblA* also results in enhanced production of specialized metabolites
713 in other streptomycetes, such as *Streptomyces ansochromogenes*, *Streptomyces*
714 *glaucescens*, *Streptomyces roseosporus* and *Streptomyces sp.* C4412 as well as in
715 *Pseudonocardia* ²⁷¹⁻²⁷⁶, and should therefore be considered as a general approach to
716 achieve enhanced production of cryptic antibiotics in a given strain. It is yet unclear how
717 WblA controls antibiotic production.

718

719 **9. AUTOREGULATORS AND THE CONTROL OF ANTIBIOTIC PRODUCTION**

720 Bacteria communicate with each other through production of small extracellular molecules,
721 called bacterial hormones or autoregulators. After the discovery of the gamma-
722 butyrolactone A-factor (2-isocapryloyl-3*R*-hydroxymethyl- γ -butyrolactone), produced by *S.*
723 *griseus*, many more bacterial hormones have been identified, such as GBLs similar to A-
724 factor, furans, gamma-butenolides and PI-factor. In general, these signalling molecules
725 are active in nanomolar concentrations and diffuse readily from one actinomycete to
726 another, thereby affecting development and antibiotic production. GBL production is most
727 likely not species-specific, as different species can produce the same GBL, suggesting
728 extensive interspecies communication and 'eavesdropping'. Antibiotics may also function
729 as signalling molecules, thereby induce antibiotic activity and/or resistance, and again in a

730 more general fashion, affecting a broad range of hosts. Thus, the usage of bacterial
731 hormones or antibiotics is an important factor in the discovery of novel antibiotics, as well
732 as co-culturing micro-organisms (recently reviewed in ²⁷⁷).

733

734 **9.1. The gamma-butyrolactone regulatory system in *S. coelicolor* and *S. avermitilis***

735 Enzymes responsible for the synthesis of gamma-butyrolactones (GBLs) in
736 streptomycetes are identifiable through their homology to the A-factor synthetase AfsA of
737 *S. griseus* ⁹¹. The orthologue of AfsA is encoded by *scbA* (SCO6266) within the *cpk* gene
738 cluster responsible for the production of the yellow compound coelimycin P1 ²⁷⁸. ScbA is
739 required for the production of the GBLs of *S. coelicolor*. This strain produces 8 different
740 GBLs (SCB1-8). The structure of these molecules have recently been solved after they
741 were overproduced in the super host M1152 ²⁷⁹. Deletion of *scbA* resulted in the
742 overproduction of Act and Red biosynthesis and reduced *cpk* expression ²⁸⁰ Divergent to
743 *scbA* lies *scbR* (SCO6265), which encodes a transcription factor that appears to activate
744 transcription of *scbA* as well as a repressor of its own transcription and that of *cpkO*
745 (*kasO*), which encodes the CSR of the coelimycin BGC cluster, provided GBL is not bound
746 by ScbR ^{142, 141}. It also positively regulates CdaR, the CSR of the Cda BGC. Deletion of
747 *scbR* resulted in reduced Act, Red and Cda production and increased coelimycin P1
748 production ¹⁴³. The regulation of *scbA* is complex, with no fewer than five *scbR* paralogues
749 in *S. coelicolor* ²⁷⁷, one of which *scbR2* (SCO6286) is also encoded within the coelimycin
750 BGC ²⁸¹. The reader is referred to our previous review for more details ³³.

751 ScbR2 is highly similar to ScbR, but unlike ScbR it is not able to bind GBLs, and is
752 hence considered a pseudo gamma-butyrolactone receptor ^{278, 282}. Instead it binds the
753 endogenous antibiotics Act and Red and the exogenous antibiotic jadomycin B and related
754 angucyclines ^{278, 283}. Interestingly, addition of non-endogenous jadomycin B from *S.*
755 *venezuelae* releases ScbR2 from the promoters of *redD* and *adpA* in *S. coelicolor*, leading

756 to accelerated Red production and morphological differentiation. ScbR2 probably has a
757 greater effect on secondary metabolism than ScbR. Deletion of *scbR2* abolishes Act, Red
758 and Cda production and induced coelimycin production^{281, 283}. Like ScbR, ScbR2 directly
759 represses *cpkO*²⁷⁸. ScbR2 is also a repressor of *scbA*, and acts both directly and
760 indirectly on antibiotic production²⁸². ChIP-seq showed that ScbR and ScbR2 have many
761 shared targets genes related to primary and secondary metabolism^{143, 284}. Both directly
762 act on *afsK* and on genes involved in malonyl-CoA synthesis and hence precursor supply
763 for polyketide natural products. Interestingly, the TetR-like proteins ScbR and ScbR2 can
764 also bind as heterodimers, and co-immunoprecipitation of ScbR2 and ScbR revealed that
765 only the ScbR-ScbR2 heterodimer can control SCO5158, which encodes an
766 uncharacterized protein²⁸⁵. Such heterodimer formation is not unique, and was previously
767 proposed for the gene products of *mmfR* and *mmyR* of the methylenomycin BGC²⁸⁶.

768 *S. avermitilis* contains three GBL-like receptors encoded by genes that are located
769 in a single locus, namely *aveR1*, *aveR2* and *aveR3*. This locus also contains the genes
770 *aco* and *cyp17* required for avenolide biosynthesis. The bacterial hormone avenolide
771 increases avermectin production in a dose-dependent manner when added in nanomolar
772 concentrations to an *aco* deletion mutant²⁸⁷. The AveR1 protein was identified as its
773 cognate receptor²⁸⁸. Deletion of *aveR1* or addition of avenolide did not influence
774 avermectin production, but increased avenolide production. An explanation for the latter
775 might be that the threshold that is required for avermectin production has already been
776 reached at the start of growth. This led to the suggestion that AveR1 acts as a repressor in
777 the early stages of growth²⁸⁹. AveR1 represses its own transcription and that of *aco*²⁸⁹.

778 AveR2 is a pseudo GBL-receptor that represses the transcription of *aveR*, encoding
779 the positive CSR of the *ave* cluster²⁹⁰. Additionally, AveR2 represses *aco* and *cyp17*, and
780 controls genes involved in primary metabolism, ribosomal protein synthesis and stress
781 responses. Such an extended regulon is reminiscent of ScbR2 (see above), and it is

782 important to note that both regulators can bind endogenous and exogenous antibiotics.
783 Indeed, the affinity of AveR2 for DNA is influenced by avermectins and also by the
784 exogenous antibiotics jadomycin B and by aminoglycosides. Thus, we note that such
785 pseudo-GBL receptors should be considered as important pleiotropic regulators²⁹⁰.

786 AveR3 shows similarity to autoregulator receptors and activates *aveR* transcription
787 of the avermectin BGC, and indirectly also filipin biosynthesis^{291, 292}. Interestingly, deletion
788 of *aveR3* resulted in the discovery of the cryptic natural product, phthoxazolin A, a
789 cellulose synthesis inhibitor that shows activity against plant pathogenic oomycetes. The
790 fact that GBL-mediated regulatory systems control cryptic genes in both *S. coelicolor* and
791 *S. avermitilis* makes them candidate targets for drug discovery.

792

793 **9.2. GBL-receptors and antibiotic production in other streptomycetes**

794 The examples of *S. coelicolor* and *S. avermitilis* suggest that the presence of genes for
795 GBLs and their receptor proteins may serve as beacons for cryptic BGCs. Similarly, the
796 BGCs for the angucyclines jadomycin B (from *S. venezuelae*) and auricin (from *S.*
797 *aureofaciens*) and also contain genes for GBL synthases and their cognate receptors²⁹³,
798²⁹⁴. The gene *jadR3* harboured within the jadomycin B BGC encodes a putative GBL
799 receptor located upstream of the GBL synthase genes *jadW123*. The product of this GBL
800 synthase system is SVB1, which is identical to the GBL SCB3, produced by *S. coelicolor*.
801 In *S. venezuelae*, only *JadW2* is required for jadomycin production²⁹⁴. Nevertheless,
802 deletion of *jadW1* abolishes both jadomycin B and chloramphenicol production under
803 conditions that are known to be favourable for production of these antibiotics²⁹⁵. *JadR3* is
804 an autorepressor and also represses *jadW1* transcription, and thereby represses
805 jadomycin B production²⁹⁴. The auricin BGC of *S. aureofaciens* is controlled by the GBL
806 synthase *SagA* and its cognate receptor *SagR*, and again the genes encoding these
807 proteins are located directly next to the biosynthetic genes. Deletion of *sagR* results in

808 early but reduced auricin production, while deletion of *sagA* abolishes auricin production,
809 establishing their key role in controlling auricin biosynthesis. In contrast to other GBL
810 receptor proteins, SagR does not auto-regulate its own transcription, but instead *sagR* and
811 *sagA* are repressed by the CSR Aur1R²⁹³.

812 Further on the theme, the production of indigoidine (a blue-pigmented compound),
813 of nucleoside antibiotics (showdomycin and minimycin) and of D-cycloserine by *S.*
814 *lavendulae* FRI-5 is controlled by the bacterial hormone IM-2 and its cognate receptor
815 FarA^{296, 297}. Supplementation of culture media with IM-2 enhances production of
816 indigoidine, but abolishes production of D-cycloserine²⁹⁶. FarA inhibits its own expression
817 and activates the expression of FarX, the protein required for IM-2 biosynthesis. The
818 genes encoding FarA and FarX are located on a regulatory island spanning 12.1 kb²⁹⁸.
819 This island contains the genes *farA-E*, *farR1-5* and *farX*²⁹⁸. FarA negatively regulates its
820 own expression and the expression of *farR1* (which encodes an orphan response
821 regulator), *farR2* (for a pseudo-GBL receptor), *farR4* (for a SARP regulator)²⁹⁹, *farB* (for a
822 structural protein)²⁹⁸. Since *farR3* and *farR4* can be transcribed both as monocistronic and
823 bicistronic mRNA, it appears that *farR3* is also a target of FarA²⁹⁹. FarR2 is a pseudo-GBL
824 receptor that positively regulates the production of indigoidine, but negatively regulates the
825 expression of the *far* regulatory genes in the regulatory island, including the expression of
826 *farX*³⁰⁰. Similarly, FarR3 positively regulates the production of indigoidine²⁹⁹, but in both
827 cases the control is most likely indirect^{300, 301}. The SARP regulator FarR4 represses IM2
828 biosynthesis²⁹⁹. which offers a unique example of a SARP regulator that acts at the front
829 instead of the end of a regulatory cascade²⁹⁹.

830 The complex regulatory network of the “pristinamycin supercluster” of *S.*
831 *pristinaespiralis* is also under the control of a GBL-receptor. Pristinamycin is a mixture of
832 two compounds, including the cyclohexanedepsipeptide pristinamycin I (PI) and the poly-
833 unsaturated macrolactone pristinamycin II (PII) that are produced in a 30:70 ratio. The

834 mixture of pristinamycin is significantly more active against pathogenic bacteria than PI
835 and PII separately ³⁰². PI is synthesized by non-ribosomal peptide synthetases (NRPS)
836 and PII by hybrid polyketide synthases (PKS)/NRPS ³⁰³. The genes required for PI and PII
837 production are not arranged in a single BGC, but are heterogeneously divided over a 210
838 kb genomic region whereby the biosynthetic genes are interspersed by a cryptic BGC ³⁰³.
839 These characteristics of the BGC and the fact that the cluster contains seven genes
840 encoding CSRs makes the regulation of pristinamycin biosynthesis very complex ³⁰⁴.
841 These CSRs include the GBL-receptor SpbR, two TetR-like regulators (PapR3 and
842 PapR5), three SARP regulators (PapR1, PapR2, PapR4) and a response regulator
843 (PapR6) ^{303, 304}. The regulatory cascade starts with the release of SpbR from the DNA
844 when its ligand reaches a critical concentration ³⁰⁴. The pristinamycin BGC is under the
845 direct control of the SARP regulators PapR1, PapR2 and the response regulator PapR6
846 ³⁰⁴. PapR2 is most likely the master regulator of the pristinamycin BGC, as this is the only
847 regulator that is fully required for pristinamycin biosynthesis ³⁰⁴. The regulatory genes that
848 directly control the pristinamycin BGC are repressed by the TetR-regulator PapR5 ^{304, 305}.
849 PapR5 shows similarity to pseudo-GBL receptors, suggesting that perhaps pristinamycin
850 and/or biosynthetic intermediates act as ligands for PapR5 and may thereby control the
851 level of pristinamycin ³⁰⁴. Similar as to other regulatory networks, the GBL-receptor is not
852 the first regulator in the regulatory cascade, since SpbR is positively regulated by an AtrA
853 (SSDG_00466) regulator outside the BGC. AtrA in turn positively controls the transcription
854 of PapR5 ³⁰⁵. Thus, the pristinamycin BGC is subject to complex and multi-level control,
855 several elements of which deserve further investigation, so as to unravel the full regulatory
856 network.

857

858 **10. EMERGING THEMES IN THE CONTROL OF ANTIBIOTIC PRODUCTION IN**
859 **ACTINOBACTERIA**

860 Besides the usual suspects, less well-studied genera of Actinobacteria (often referred to
861 as rare Actinobacteria) also produce a wide range of natural products, and insights into
862 their molecular regulation is important from the perspective of drug discovery and
863 production improvement. Culture collections housed by biotechnology companies and
864 research institutes possess several rare Actinobacteria, including *Micromonosporaceae*,
865 *Streptosporangiae*, *Pseudonocardiaceae*, *Nocardiaceae*, and *Thermomonosporaceae*,
866 and many other rare and unclassified species that have yet to be explored³⁰⁶⁻³⁰⁹. In
867 recent years, interest in strains isolated from marine environments and other ecological
868 niches such as plants and insects has grown because they offer a rich new microbial
869 source for NP discovery^{35, 310, 311}. The regulation of natural product biosynthesis by rare
870 Actinobacteria is poorly characterised, because many of them are genetically intractable
871 and limited genetic tools are available. As the cell wall structure between Actinobacteria
872 often varies and is different from that of streptomycetes, preparation of protoplasts (and
873 regeneration) typically requires different methods³¹². A protocol to prepare protoplasts of
874 *Planobispora rosea*, the producer of the thiazolyl peptide antibiotic GE2270 that targets
875 elongation factor EF-Tu³¹³ was applied to different rare Actinobacteria³¹². This protocol
876 demonstrated the applicability of both lysozyme and mutanolysin (from *S. globisporus*) to
877 produce protoplasts from these industrially important strains³¹². Other issues that need to
878 be solved for genetic manipulation of rare actinobacteria include identification of suitable
879 origins of replication for plasmids³¹⁴, the methylation pattern of the DNA^{315, 316} and the use
880 of specific promoters for expression^{317, 318}. Many of these technical difficulties can in
881 principle be circumvented by the use of expression of a BGC in a heterologous host.
882 Expression of the BGC for GE2270 of *P. rosea* in *S. coelicolor* M1146 allowed the study of
883 its regulation³¹⁹. Deletion of *pbtR*, encoding a TetR-family regulator, abolished the
884 production of GE2270. Similarly, the BGC for taromycin A from *Saccharomonospora* sp.
885 CNQ490 was also expressed in *S. coelicolor* M1146 to allow its genetic manipulation.

886 Deletion of *tar20*, encoding a LuxR regulator of the taromycin BGC, increased the
887 production of the compound in the heterologous strain ³²⁰. Heterologous expression of a
888 BGC may often be suitable to study the function of CSRs within a BGC, but for
889 understanding of the global regulatory network and the ecological responses that control
890 the BGC of interest, it is necessary to study the BGC in its natural host. In a number of
891 Actinobacteria, the molecular regulation of antibiotic production has been studied.
892 Especially in strains that produce clinically important antibiotics, such as glycopeptide
893 producers. It appears that the rare Actinobacteria that have been studied indeed contain
894 similar regulators as *Streptomyces* and therefore we expect that most of the control
895 mechanisms of antibiotic production are similar. Below the control of antibiotic production
896 in a number of Actinobacteria is discussed and compared to that of *Streptomyces*.

897

898 **10.1. Control of glycopeptide biosynthesis**

899 The glycopeptide antibiotics vancomycin and teicoplanin are important last line of defence
900 antibiotics that are used to treat infections associated with multi-drug resistant Gram-
901 positive bacteria ^{321 322}. Their target is the peptidoglycan precursor lipid II, thereby
902 inhibiting synthesis of the bacterial cell wall ³²³. Vancomycin is produced by *Amycolatopsis*
903 *orientalis* and teicoplanin by *Actinoplanes teichomyceticus* ^{324, 325}. Other well-studied
904 members include the precursor of dalbavancin, A40926 produced by *Nonomuraea sp.*
905 ATCC39727 ³²⁶, balhimycin produced by *Amycolatopsis balhimycina* ³²⁷, and the
906 sugarless glycopeptide A47934 produced by *S. toyocaensis* ³²⁸. A comparison of the
907 BGCs for these compounds (*tei* for teicoplanin, *bal* for balhimycin and *dbv* for A40926)
908 and their control is presented in Fig. 6. Members of the glycopeptides share a
909 heptapeptide core, which is synthesized by non-ribosomal peptide synthetases (NRPS),
910 with further modifications such as cross-linking, methylation, halogenation glycosylation
911 or attachment of sulphur groups ^{322, 329}. Glycopeptides bind to the D-alanyl-D-alanine(D-

912 ala-D-ala) terminus of the growing lipid attached peptidoglycan chain on the outside of the
913 cytoplasmic membrane and thereby prevent the binding of transpeptidases that create
914 the cross-links between the polysaccharides, required for cell wall integrity³²³.

915 The BGCs of these antibiotics are typically controlled by CSRs of the StrR and
916 LuxR families³³⁰⁻³³². The teicoplanin BGC spans 89 kb and includes five regulatory genes,
917 *tei2*, *tei3*, *tei15**, *tei16** and *tei31**^{324, 325}. *Tei2* and *Tei3* show high homology with the
918 VanR/VanS system of *S. coelicolor*^{333, 334} and are involved in the control of teicoplanin
919 resistance. The genes *tei15** and *tei16** encode members of the StrR and LuxR family
920 regulators, respectively. Overexpression of *Tei15** results in 30-40-fold increase in
921 teicoplanin biosynthesis^{332, 335}. *Tei15** is the primary CSR, and directly controls the
922 transcription of the regulatory genes *teiA* for the NRPS module, *tei2** (which encodes a
923 deacetylase), *tei16**, *tei17** involved in Dpg synthesis and *tei27** (for an unknown protein).
924 *Tei15** also controls the expression of the LuxR family regulator *Tei16** and the SARP
925 family regulator *Tei31**. The targets of *Tei16** and *Tei31** in the teicoplanin cluster remain
926 unknown, although *Tei16** does positively control teicoplanin production³³². *Tei15** does
927 not show autoregulation, in contrast to its orthologue *BbR* in the balhimycin BGC^{331, 332}.
928 See Fig. 6.

929 The dalbavancin BGC of *Nonomuraea* sp. ATCC39727 contains four regulatory
930 genes, namely *dbv3*, *dbv4*, and the TCS *dbv6* and *dbv22* for the control of resistance (Fig.
931 6). *Dbv4* (similar to StrR and *Tei15**) is the likely CSR, and is expressed under phosphate-
932 limiting conditions, while *Dbv3* is a LuxR-type regulator similar to *Tei16**. Both *Dbv3* and
933 *Dbv4* are required for A40926 production³³⁰. *Dbv3* controls the transcription of *dbv4*, as
934 well as genes for the biosynthesis of 4-hydroxyphenylglycine, the heptapeptide backbone,
935 and for glycosylation and export. However, similar to the situation for *Tei16** in the
936 teicoplanin BGC, no common regulatory elements were identified in the promoter regions
937 of the *Dbv3*-controlled genes, and control could therefore be indirect³³⁰. *Dbv4* is directly

938 involved in the regulation of genes involved in 3,5-dihydroxyphenylglycine, cross-linking,
939 halogenation, glycosylation and acylation³³⁰. Dbv4 and the Dbv4 regulon are repressed by
940 phosphate, whereas Dbv3 and its regulon are not. No Pho-boxes were identified upstream
941 of the *dbv4* genes, suggesting the phosphate repression is indirect³³⁶.

942 The glycopeptide balhimycin is produced by *Amycolatopsis balhimycina* (formerly
943 *Amycolatopsis mediterranei*). The balhimycin BGC has a simpler control system with three
944 regulatory genes, namely the VanR/VanS TCS for resistance and the StrR-like regulator
945 Bbr (Fig. 6). Bbr binds to a consensus sequence (GTCCAR(N)₁₇TTGGAC) that is found
946 within the promoter for its own transcription, the putative ABC transporter gene *tba*, *oxyA*
947 for a P450 monooxygenase, *dvaA* involved in dehydrovancosamine synthesis and the
948 putative sodium proton antiporter gene *orf7*³³¹. In the three glycopeptide BGCs the StrR
949 CSR binds to the consensus sequence that is conserved in the intergenic regions of the
950 glycopeptide BGCs, although the target sequence may vary and deviate from the
951 consensus^{329, 331, 332, 336}. Although these three BGCs are organised in a similar manner
952 and contain regulatory genes, the mechanism of regulation differs between them, and
953 therefore making assumptions about the regulatory network based on bioinformatics alone
954 is not sufficient³³⁰. In *S. griseus*, StrR is positively controlled by the pleiotropic regulator
955 AdpA. However, overexpression of the putative *adpA* gene of *A. balhimycina* did not
956 induce antibiotic production, although heterologous expression of this regulator in *S.*
957 *coelicolor*, *S. ghanaensis* and several soil Actinobacteria was successful³³⁷. Vancomycin
958 biosynthesis and its control are well understood, but the role of StrR regulator in the BGC
959 (AORI_1475) has not been elucidated.

960 Since most glycopeptide BGCs contain a StrR-like positive regulator, over-
961 expression of the corresponding gene is a logical generic strategy to induce the
962 expression of (cryptic) glycopeptide BGCs. A good example is the production of ristomycin
963 A in *Amycolatopsis japonicum*. This strain is known for the production of (S,S)-

964 ethylenediamine disuccinic acid [(S,S)-EDDS], the biodegradable isoform of EDTA
965 (section 7) . Under standard laboratory conditions this strain does not produce antibiotics,
966 but over-expression of the StrR orthologue in *A. japonicum* induced the production of
967 ristomycin A, which is used for the diagnosis of von Willebrand disease and Bernard-
968 Soulier syndrome ³³⁸.

969

970 **10. 2. Control of glycopeptide resistance**

971 Bacteria that are resistant against glycopeptide antibiotics replace the D-alanine for D-
972 lactate as the terminal residue of the peptide chain of the peptidoglycan. As the affinity of
973 the glycopeptide for the latter is a lot lower than for D-ala-D-ala, binding of the
974 glycopeptide is prevented ^{339, 340}. The glycopeptide BGCs contain genes that encode
975 homologues of the VanR/VanS TCS that governs glycopeptide resistance.

976 *S. coelicolor* is resistant against vancomycin and this resistance is conferred by
977 genes that are similar to the ones present in vancomycin resistant enterococci ^{333, 334}. The
978 resistance cluster of *S. coelicolor* is organized in four transcription units, namely *vanRS*,
979 *vanJ*, *vanK* and *vanHAX*. The latter encode the enzymes required for biosynthesis and
980 incorporation of D-lac in the peptide moiety of the PG. All transcription units are regulated
981 by VanRS ³³³. Binding of vancomycin by the N-terminal part of VanS leads to its
982 autophosphorylation, and this phosphate is then transferred to the N-terminal receiver
983 domain of VanR, thereby activating its C-terminal DNA binding effector domain. This
984 results in expression of the resistance genes. In the absence of vancomycin VanS acts a
985 phosphatase that dephosphorylates VanR, and hence *vanS* mutants show constitutive
986 expression of vancomycin resistance ^{334, 341}. In contrast, deletion of *vanS* in *S.*
987 *toyocaensis* results in sensitivity to A47934, and it was suggested that VanR of *S.*
988 *coelicolor* is phosphorylated by other proteins while that of *S. toyocaensis* is not ³⁴².
989 Interestingly, the VanRS TCS is an important determinant of the species-specific

990 glycopeptide resistance profile. *S. coelicolor* is resistant against vancomycin and A47934,
991 but sensitive to teicoplanin, while *S. toyocaensis* is only resistant against A47934⁸.
992 Exchanging the VanRS TCSs between the two *Streptomyces* strains is sufficient to switch
993 the resistance profile⁸. Surprisingly, expression of the VanR orthologue of *A. balhimycina*
994 (VnIR) in *S. coelicolor* even governed resistance to teicoplanin, and led to increased
995 actinorhodin biosynthesis³⁴³. VnIR controls *vanHAX* in *S. coelicolor*, despite the fact that
996 it does not control *vanHAX* in *A. balhimycina* itself³⁴³.

997

998 **10.3. σ -factor/anti- σ -factor systems and the control of antibiotic biosynthesis**

999 An important new element of antibiotic control that was discovered in recent years is the
1000 control by σ -factors, the subunits of the RNA polymerase responsible for promoter
1001 recognition. An important example is that of the control of lantibiotics. Lantibiotics are
1002 ribosomally synthesized, post translationally modified peptide antibiotics (RiPPs;³⁴⁴). The
1003 best known lantibiotic is the food-preservative nisin, produced by *Lactococcus lactis* and
1004 discovered as early as 1928³⁴⁵. Lantibiotics are synthesized as a prepropeptide encoded
1005 by a precursor gene generally referred to as *lanA*. This propeptide is post-translationally
1006 modified via intramolecular lanthionine bridges that are formed between unusual amino
1007 acids to yield the mature peptide³⁴⁶. Nisin and several other lantibiotics target the
1008 pyrophosphate linkage component of the cell-wall precursor lipid II. As this target is
1009 different from that of the clinically used antibiotic vancomycin, there is no cross-resistance
1010 with glycopeptides, making them interesting new antibiotics for the treatment of methicillin
1011 resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE)³⁴⁷.
1012 Screening a library of 120,000 chemical extracts derived from 40,000 Actinobacteria for
1013 activity against cell-wall biosynthesis by Vicuron Pharmaceuticals identified five novel
1014 lantibiotics, including microbisporicin (also known as NAI-107) and planosporicin,
1015 produced by *Microbispora corallina* and *Planobispora alba*, respectively^{348, 349}. The control

1016 of the BGCs for microbisporicin (*mib* in *M. corallina* and *mlb* in *M. ATCC-PTA-5024*) and
1017 for planosporicin (*psp*) have been studied in detail ³⁵⁰⁻³⁵². The BGCs have a gene for an
1018 extracytoplasmic function (ECF) σ -factor /anti- σ -factor complex (MibX/MibW for
1019 microbisporicin and PspX/PspW for planosporicin). ECF σ factors mediate responses to
1020 extracellular signals and stress or steps in morphological differentiation ^{353, 354}, but their
1021 involvement in the control of antibiotic production was only recognized recently. The
1022 microbisporicin and planosporicin BGCs also contain a gene for a regulator with a LuxR-
1023 like C-terminal domain. Herein, we use microbisporicin biosynthesis as the example for
1024 both BGCs, see Fig. 7 for an overview of its control. The BGC is controlled by its own
1025 production by a feed-forward mechanism: deletion of *mibA* results in decreased
1026 transcription of the other *mib* genes, while growth of *mibA* mutant colonies adjacent to
1027 wild-type microbisporicin-producing colonies restored *mib* transcription ^{351, 352, 355}. This
1028 effect is specific, since microbisporicin cannot induce the production of planosporicin by
1029 *Planobispora alba* ³⁵¹. The *mib* cluster includes six transcription units, for synthesis,
1030 modification, proteolysis, export, immunity and regulation, and all except the *mibA*
1031 structural gene contain the ECF σ -factor promoter motif (GACC-N15-GCTAC) that is
1032 recognized by MibX ^{350, 352, 355} (Fig. 7). The promoter of *mibA* is controlled by MibR; in turn,
1033 transcription of *mibR* depends on MibX and is enhanced by the stringent response.
1034 Indeed, deletion of *relA* in *M. corallina* abolishes microbisporicin production. Thus, a
1035 complex regulatory network ensures the correct timing of microbisporic biosynthesis,
1036 which is induced by both nitrogen starvation and the ensuing stringent response, which
1037 activates MibR expression and hence the expression of the (non-toxic) precursor peptide.
1038 This precursor is then exported and processed to yield the active antibiotic ³⁵⁰. Under
1039 repressing conditions, MibX is recruited by the membrane bound anti sigma factor MibW,
1040 thereby shutting down the biosynthetic pathway. Microbisporicin production also directly
1041 depends on the developmental programme, with reduced expression in *bld* mutants,

1042 similarly to the biosynthesis of the morphogenic lantibiotic-like morphogen SapB in *S.*
1043 *coelicolor*³⁵⁶. For a detailed overview on the regulation of RiPPs in Actinobacteria and
1044 other bacterial genera, we refer the reader a recent review³⁵⁷.

1045 Involvement of σ factors in the control of antibiotic production is not exclusive to
1046 lantibiotic BGCs. SigT regulates Act production in *S. coelicolor* via *relA* in response to
1047 nitrogen starvation, which links nitrogen stress to secondary metabolism³⁵⁸. In *S. albus*,
1048 the ECF σ^{AntA} controls the synthesis of the antimycin precursor, 3-formamidosalicylate³⁵⁹,
1049 σ^{25} , and σ^{25} differentially controls the biosynthesis of oligomycin and of the important anti-
1050 helminthic drug avermectin in *S. avermitilis*³⁶¹. Antimycin is a mitochondrial cytochrome c
1051 reductase inhibitor produced by diverse actinobacteria. σ^{AntA} was the first example of
1052 regulation of antibiotic production by a cluster-situated ECF σ factor in *S.* species and it
1053 was recently shown that this is likely to be a conserved strategy of regulation for more than
1054 70 antimycin BGCs³⁶². Unlike other ECFs, which are controlled by an anti- σ factor that is
1055 unable to maintain an inactive complex in the presence of cognate stimulus, σ^{AntA} is an
1056 orphan and is not controlled by such a factor. Instead, evidence to date suggests that σ^{AntA}
1057 is controlled by Clp proteolysis³⁵⁹. The involvement of σ -factor genes in the control of
1058 antibiotic production is a new concept, and in particular the presence of σ factor genes
1059 within BGCs may function as beacons to identify BGCs in genome mining.

1060

1061 **10.4. Regulation of antibiotic production in *Salinispora***

1062 Recently, studies have also been dedicated to the regulatory network of natural product
1063 biosynthesis in the marine actinomycete *Salinispora*. *Salinispora* is an obligate marine
1064 actinomycete and most of the isolates are derived from marine sediments. The genus
1065 knows three different species, under which *S. pacifica*, *S. tropica* and *S. arenicola*³⁶³. The
1066 compounds that were discovered from this genus are predominantly new and therefore
1067 this genus is a good example of the concept that new genera derived from remote areas

1068 are a good source for the discovery of novel natural products ³⁶³. One of these studies
1069 reveals that in *S. tropica* CNB-440, a LuxR-type regulator positively regulates the
1070 biosynthesis of the important natural product salinisporamide A, a proteasome inhibitor
1071 that is in stage 1 of clinical trials of anti-cancer treatment. This regulator controls the genes
1072 involved in the biosynthesis of the salinisporamide A precursor chloroethylmaloyl-CoA,
1073 and thereby specifically regulates the production of salinisporamide A and not of other
1074 salinosporamides that are produced by *S. tropica* CNB-440 ³⁶⁴.

1075 In the genus *Salinispora* an important concept for the study of cryptic gene clusters
1076 was revealed ³⁶⁵. Transcriptomic comparison of the *Salinispora* strains *S. pacifica* CNT-
1077 150, *S. tropica* CNB-440, *S. arenicola* CNS-205 and *S. arenicola* CNS-991 revealed that
1078 BGCs common between different strains are not necessarily controlled in the same way
1079 and could be active in one while silent in another. Such strain-specific silencing of a BGC
1080 was explained by mutation of regulatory genes. Indeed, an orphan BGC in *S. pacifica*
1081 (STPKS1) was expressed normally, while its counterpart in *S. tropica* was silent due to the
1082 lack of the AraC-family CSR, which was replaced by a transposase. Interestingly, this
1083 silent gene cluster is conserved throughout the *S. tropica* clade, which suggests that either
1084 this BGC is permanently silenced or that another regulator is involved in the control of the
1085 BGC. The BGC for the enediyene PKS1A was silent in CNS-991 and expressed in CNS-
1086 205. Comparative genomics and transcriptomic data revealed that a σ factor upstream of
1087 the BGC was expressed in *S. arenicola* CNS205, but not in CNS991. Differential
1088 expression of this σ factor was proposed be a consequence of its different chromosomal
1089 location in the two strains. The BGC for the black spore pigment was present in all four
1090 *Salinispora* strains, but the full BGC was only expressed by *S. tropica* CNB-440 and *S.*
1091 *pacifica* CNT-150, whereas only a subset of the genes within the gene cluster was
1092 expressed in the two *S. arenicola* strains. The spore pigment BGCs that were entirely
1093 expressed contained one or two *luxR* genes, whereas the partially expressed BGC

1094 contained small genes encoding hypothetical proteins of unknown function. The *sta* gene
1095 cluster for staurosporine was also differentially expressed between the four *Salinispora*
1096 strains, but all strains contained the *malT* gene for the CSR. Finally, the fact that a BGC
1097 (NRPS4) was expressed in *S. arenicola* and *S. pacifica*, but not in *S. tropica* was
1098 explained by the lack of a xenobiotic response element in *S. tropica*³⁶⁵. Further genetic
1099 analysis of these interesting examples is required to fully understand the regulatory
1100 mechanisms for these BGCs. The differential expression of gene clusters between
1101 different species suggests that one feasible approach to the problem of silent gene
1102 clusters may be to look for the same (or highly similar) gene cluster in related
1103 actinobacteria, and see if the cluster is expressed there. With the ever-growing genome
1104 sequence information, this approach is becoming increasingly feasible, and is particularly
1105 attractive in strains that are not genetically tractable.

1106

1107 **10.5. Regulation of rifamycin biosynthesis in *Amycolatopsis mediterranei***

1108 Recently, the molecular regulation of the rifamycin BGC was studied in *Amycolatopsis*
1109 *mediterranei*. Although rifamycin and its derivatives are the first-line anti-tuberculosis
1110 drugs, the regulation of the rifamycin BGC was only studied recently. Deletion of *glnR*
1111 influences the biosynthesis of rifamycin, although this control is indirect³⁶⁶. The LuxR-type
1112 regulator RifZ, encoded by the last gene in the gene cluster, positively controls all of the
1113 operons in the rifamycin BGC³⁶⁷. The rifamycin BGC also encodes a TetR-family
1114 repressor (RifQ), which represses rifamycin biosynthesis and efflux. Deletion of *rifQ*
1115 resulted in increased production of rifamycin, while accumulation of rifamycin B lowered
1116 the affinity of RifQ for its target sequences³⁶⁸. This system is consistent with what is
1117 known for other TetR-family regulators that control natural product biosynthesis.

1118

1119 10.6. GBL-receptors and antibiotic production in Actinobacteria other than**1120 *Streptomyces***

1121 GBL-like molecules are produced by many actinobacteria, including the industrial
1122 important strains *A. teichomyceticus* (producer of teicoplanin), *A. mediterranei* (produces
1123 rifamycin), and *Micromonospora echinospora* (produces gentamicin)³⁶⁹. The exact
1124 structures of the GBL molecules produced by these strains are unknown, but the type of
1125 GBL that is produced could be determined using binding assays with tritium-labeled GBL
1126 molecules as ligands^{369, 370}. These binding assays confirmed that *A. teichomyceticus*
1127 produces a GBL similar to virginiae butenolide (VB) derived from *S. virginiae*. The strains
1128 *A. mediterranei* and *M. echinospora* produce a GBL similar to IM-2, derived from *S.*
1129 *lavendulae* (see section 9.2)³⁶⁹. In the rifamycin producer *A. mediterranei*, four genes that
1130 encode GBL-receptor paralogues are present, namely *bamA1-bamA4*³⁷¹. All four receptor
1131 proteins can bind GBLs derived from *Streptomyces*, including VB from *S. virginiae* and
1132 SCB1 from *S. coelicolor*. Only BamA1 was shown to bind the IM-2 GBL, an autoregulator
1133 produced by *A. mediterranei* itself^{369, 371}.

1134 *Kitasatospora setae*, a member of a genus closely related to *Streptomyces*,
1135 harbours several GBL-receptors^{264, 372}. *K. setae* produces bafilomycins A1 and B1. These
1136 macrolides specifically inhibit vacuolar H⁺-ATPases and are used in studies of molecular
1137 transport in eukaryotes. The genome of *K. setae* contains three genes that are similar to
1138 GBL-receptors, namely *ksbA*, *ksbB* and *ksbC*³⁷³. KsbA binds ³H-labeled SCB1, and
1139 deletion of *ksbA* increases bafilomycin biosynthesis³⁷². Conversely, KsbC indirectly
1140 represses bafilomycin biosynthesis, perhaps via the activation of the gene for the
1141 autoregulator KsbS4³⁷³. KsbC also indirectly activates the production of kitasetaline, a β -
1142 carboline alkaloid, and of the kitasetaline derivative JBIR-133³⁷³.

1143 Interestingly, *Rhodococcus jostii*, a genus of the *Nocardiaceae* produces the GBL
1144 (called RJB) that is structurally identical to a precursor of SCB2 (6-dehydro SCB2)

1145 produced by *S. coelicolor*, and can bind to the *S. coelicolor* GBL receptor ScbR³⁷⁴. This
1146 suggests cross-family communication mediated by GBLs in the natural environment. The
1147 gene for GBL biosynthesis, *gblA*, is located in a GBL BGC that is conserved between
1148 different *Rhodococcus* species. This GBL BGC also encodes a GBL-receptor protein
1149 GblR and the biosynthesis enzyme GblE, which is an NAD-epimerase/dehydratase.
1150 Genome sequencing of *R. jostii* RHA1 indicated that the strain potentially has a rich NP
1151 biosynthetic repertoire. The precise role of GBLs in the regulation of natural product
1152 biosynthesis in *Rhodococcus*, and the value of the NPs these Actinobacteria can
1153 produce, merit further investigation.

1154

1155

1156 **11. OUTLOOK**

1157 Over the last decade it has become increasingly clear that *Streptomyces* species and
1158 other antibiotic-producing Actinobacteria produce only a small percentage of their
1159 secondary metabolome under laboratory conditions. Accessing the chemistry specified by
1160 this 'silent majority' - also referred to as dark matter - without a doubt holds potential for
1161 drug discovery. This untapped resource can be harnessed by both genetic and non-
1162 genetic methods which been reviewed recently³⁷⁵. The proverbial 'holy grail' in this
1163 respect is development of small molecules that can simply be added to culture media to
1164 elicit the production of all or ideally only a subset of compounds. Progress has been
1165 achieved in this area (i.e. sugar-responsive antibiotic repressors, REEs, GBLs and
1166 manipulation of C, N and P concentrations, discussed above); the molecular insights that
1167 is reviewed above can be harnessed to develop strategies to activate antibiotic production.
1168 Clearly, more work is required with the identification of other small molecules. Reporter-
1169 based methods have therefore been developed to aid detection of activated or de-
1170 repressed gene clusters^{376, 377}, and screening using small molecule libraries forms an

1171 attractive black box alternative to rational approaches that are based on molecular insights
1172 ^{378, 379}. For details on molecular, environmental and HT screening approaches to find
1173 elicitors we refer the reader to recent reviews ^{35, 380}. Elicitors are also instrumental in
1174 unsupervised metabolomics approaches, required to identify compounds in the complex
1175 metabolic matrix of microbial cultures ³⁸¹. Here, significant fluctuation of the secondary
1176 metabolome needs to be achieved, allowing statistical correlation of a given bioactivity of
1177 interest to a specific metabolite and/or a BGC. NMR- or MS-based metabolomics then
1178 facilitate the identification of the sought-after bioactive molecules ^{382, 383 384, 385}.

1179 Ultimately, the productivity of any given biosynthetic pathway is dictated by one or
1180 more CSRs. The examples provided by among others *Salinispora* show that BGCs may
1181 be silent in one species of a given genus, and active in another. Thus, with the growing
1182 wealth of genome sequence information, a promising strategy is to look for related
1183 bacteria that harbour a close relative of the gene cluster of interest. Indeed, it is not
1184 illogical to assume that over the hundreds of millions of years of evolution, the natural
1185 products specified by the BGCs have remained structurally the same or highly similar, but
1186 are expressed under different growth conditions or in response to different environmental
1187 stimuli. The functionality of most putative CSRs can be deduced bioinformatically (i.e. as a
1188 repressor or an activator). Therefore, an obvious strategy and one that is commonly
1189 employed for elicitation of poorly expressed BGCs is augmentation of endogenous
1190 regulatory system(s). For example, by deleting genes encoding repressors or over-
1191 expressing those encoding activators ^{232, 386}. This strategy depends upon the genetic
1192 tractability of the organism, but this is becoming less and less of a requirement as the
1193 cloning of large genomic fragments and their *de novo* synthesis becomes more feasible,
1194 which enables their tractability and heterologous expression in a panel of potential hosts
1195 ³⁸⁷⁻³⁸⁹. Indeed, it is now possible to completely refactor the regulation of a biosynthetic
1196 pathway by replacing native promoters with those that are constitutively expressed to

1197 increase production titres using CRISPR-Cas9 technology ³⁹⁰. Longer term, improved
1198 understanding of how secondary metabolism is controlled and the development of
1199 approaches to exploit this and/or efficient synthetic biology strategies to activate
1200 biosynthetic pathways are required in order to capitalise on the treasures beneath our feet.

1201

1202

1203 **Conflicts of interests**

1204 The authors have no conflicts to declare.

1205

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1930 **Table 1. Major families of regulators involved in the control of antibiotic production.**
 1931 Representative examples and their host and target are indicated.
 1932

Family ^Δ	Example	Host *	Control [#]	Target BGC [^] , comment	Reference
SARP	ActII-ORF4, RedD, CdaR	<i>S. coelicolor</i>	(+)	Act, Red, Cda, respectively	68
	AfsR	<i>S. coelicolor</i>	(+)	activates transcription of AfsS	138
	FarR3/ Far4	<i>S. lavendulae</i>	(+,-)	Indigoidine, nucleoside and D-cycloserine	299
StrR (ParB-Spo0J)	StrR	<i>S. griseus</i>	(+)	streptomycin	391
	Tei15*	<i>Actinoplanes teichomyeticus</i>	(+)	teicoplanin	332, 335
	Dbv4	<i>Nonomuraea sp. TCC39727</i>	(+)	A40926	330, 336
LAL	FscRI	<i>S. albus</i>	(+)	candicidin and antimycin	121
	AveR	<i>S. avermitilis</i>	(+,-)	avermectin and oligomycin	392
	Dbv3	<i>Nonomuraea sp. ATCC39727</i>	(+)	A40926	330
TetR	AtrA	<i>S. griseus</i>	(+)	Global regulator	92
	ArpA	<i>S. griseus</i>	(-)	GBL receptor, repressor of <i>adpA</i>	86
	ScbR	<i>S. coelicolor</i>	(+,-)	GBL receptor	143
AraC/XylS	AdpA	<i>S. griseus</i>		activates StrR expression	90
GntR	DasR	<i>S. coelicolor</i>	(+,-)	global regulator of antibiotic production; effector molecule is N-acetylglucosamine	95, 194
c-AMP receptor protein	Crp	<i>S. coelicolor</i>	(+)	regulator coordinating development, primary and secondary metabolism	103
Orphan RR	RedZ	<i>S. coelicolor</i>	(+)	Red	73
	GlnR	<i>S. coelicolor</i>	(+)	Act and Red	145, 151
TCS	AbsA1/AbsA2	<i>S. coelicolor</i>	(-)	Act, Red, Cda	98
	AfsQ1/2	<i>S. coelicolor</i>	(+)	Act, Red, Cda; responds to nitrogen	111
	PhoRP	<i>S. coelicolor</i>	(+,-)	Act; global regulator	129, 230
	DraR/K	<i>S. coelicolor</i>	(+,-)	Act, Red, coelimycin, responds to high concentrations of nitrogen.	113
	OsdR/K	<i>S. coelicolor</i>	(+)	Act, responds to oxygen level	112
ROK	Rok7B7	<i>S. coelicolor</i>	(+,-)	Act, Red, Cda; CCR	209, 210
σ Factor	MibX/MibW	<i>Microbispora corallina</i>	(+)	microbisporicin	350, 352
	Sigma(AntA)	<i>S. albus</i>	(+)	antimycin.	359
BldB	BldB	<i>S. coelicolor</i>	(+)	antibiotic production, development and CCR	187-189
tRNA	BldA	<i>Streptomyces species</i>		leucine-tRNA for UAA codon. Translational control of antibiotic production and	393

XRE	MmyB	<i>S. coelicolor</i>	(+)	morphogenesis methylenomycin B; controlled by furans	286, 394
Wbl (WhiB-like protein)	WblA	<i>S. coelicolor</i>	(-)	pleiotropic regulator of antibiotic production and development	395
LacI	AcrC	<i>Actinoplanes</i> sp. SE50/110	(-)	acarbose	396
LmbU		<i>S. lincolnensis</i>	(+,-)	lincomycin	397, 398
Lrp/AsnC	SCO3361	<i>S. coelicolor</i>	(+)	Act; control by amino acids	399
NsdA	<i>NsdA</i>	<i>S. coelicolor</i>	(-)	Act, Cda, Mmy	400
IcIR	NdgR	<i>S. coelicolor</i>	-	Act; dependent on amino acids.	401
MarR	DptR3	<i>S. roseosporus</i>	+	daptomycin	402

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* *Streptomyces* abbreviated with 'S.'

1935 # activation indicated by +, repression by -.

1936 ^ Act, actinorhodin; Cda, calcium-dependent antibiotic; Red, prodiginines; Mmy, methylenomycin.

1937 ^ LAL, Large ATP-binding regulators of the LuxR family (in the text mentioned as LuxR);

1938 XRE, xenobiotic response element

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Table 2. Recently discovered transcriptional regulators that control antibiotic production in *S. coelicolor*. Orthologues also studied in *S. avermitilis* or *S. venezuelae* are indicated.

Gene	ID ^	Function(s) of the regulator(s) #	Ref
Regulators known to directly control antibiotic BGCs			
<i>mtrAB</i>	SCO3013/2 SVEN2756/5	TCS; MtrA activates <i>actII-ORF4</i> and <i>redZ</i> and links their production to development.	403
<i>draRK</i>	SCO3063/2; SAV3481/0	TCS; regulator of <i>actII-ORF4</i> and <i>kasO</i> in <i>S. coelicolor</i> and of <i>olmRI</i> in <i>S. avermitilis</i> . Impacts Red and Ave production in <i>S. coelicolor</i> and <i>S. avermitilis</i> , resp.	113
	SCO3361	Lrp/AsnC family positive regulator for Act production. Binds to <i>actII-ORF4</i> (EMSA).	399
<i>crp</i>	SCO3571	Regulator of primary and secondary metabolism; activates <i>actII-ORF4</i> , <i>cdaR</i> and <i>cpkA</i> (Chip-seq).	103
<i>glnR</i>	SCO4159 SAV4042	Activator of <i>actII-ORF4</i> and repressor of <i>redZ</i> in <i>S. coelicolor</i> (EMSA). Activator of <i>aveR</i> (avermectin) and repressor of <i>olmRI/olmRII</i> (oligomycin) in <i>S. avermitilis</i> (EMSA).	152
<i>abrC1C2C3</i>	SCO4596	Atypical TCS with two kinase (C1 and C2); response regulator <i>AbrC3</i> is a transcriptional activator of <i>actII-ORF4</i> (ChIP-chip); impacts Red production.	101
<i>lexA</i>	SCO5803	Global regulator of the DNA damage response; Repressor of <i>actII-ORF4</i> (EMSA).	404
	SCO6256	GntR family regulator of antibiotic production. Direct activator of <i>cdaR</i> and indirect repressor of Act production (EMSA).	405
<i>scbR2</i>	SCO6286	Activator of <i>actII-ORF4</i> , <i>redD</i> , <i>redZ</i> and <i>cdaR</i> ,	143

		repressor of <i>cpkO</i> and SCO6268 (<i>cpk</i> cluster) (Chip-seq, EMSA).	
Regulators in pathway with missing link to antibiotic gene clusters			
<i>ohkA</i>	SCO1596 SAV6741	Orphan HK; plays global role in antibiotic biosynthesis, by influencing precursor supply, pleiotropic and pathway-specific antibiotic regulators.	406
<i>abrA1A2</i>	SCO1744/5	TCS; represses Act, Red and Cda production and morphological differentiation.	237
	SCO2140	Lrp/AsnC family protein. Indirectly regulates ACT and CDA production or cooperate with other transcriptional regulators involved in production of these antibiotics (EMSA).	407
<i>aor1</i>	SCO2281	Orphan response regulator; upregulates Act, Red and Cda production and downregulates <i>sigB</i> , thus linking antibiotic production to osmotic stress response.	408
<i>stgR</i>	SCO2964	LTTR; Negative regulator for Act and Red production through upregulation of <i>actII-ORF4</i> and <i>redZ</i> , respectively. Exact regulatory cascade remains unknown.	409
<i>sigT</i>	SCO3892	ECF sigma factor; required for normal Act production under nitrogen limitation.	358
<i>cmdABCDEF</i>	SCO4126 – SCO4131	Operon for membrane proteins; affects differentiation and causes increased production of Act.	410
<i>phoU</i>	SCO4228	Activates Act and Red production. Exact regulatory cascade unknown.	411

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^SCO, *S. coelicolor*; SAV, *S. avermitilis*; SVEN, *S. venezuelae*; See StrepDB for the full annotation (<http://strepdb.streptomyces.org.uk>).

Experimental evidence presented between brackets (EMSA, Electromobility shift assay; ChIP-Seq, chromosome immunoprecipitation combined with next-generation sequencing).

1956 **Figure 1. The PhoRP and AfsKRS systems and their interplay in regulation of**
1957 **nitrogen metabolism and antibiotic production.** Black arrows indicate activation and
1958 red bars indicate repression, cyan arrows indicate expression of genes. During growth
1959 under phosphate deplete conditions, the global regulator PhoP is activated by the
1960 membrane-bound sensor kinase, PhoR. Activated PhoP acts directly upon BGCs by
1961 modulating expression of CSRs or other transcription factors, such as *glnR*, which controls
1962 expression of nitrogen metabolism genes and *afsS*, part of AfsKRS regulatory system.
1963 PhoP may directly inhibit expression of nitrogen assimilation genes and has an indirect
1964 negative impact (through ScbAR system) on expression of *afsK*. KbpA and S-adenosyl-L-
1965 methionine (SAM) can also modulate the activity of AfsK. The membrane associated
1966 kinase, AfsK, in turn, activates AfsR. AfsR interacts with the PhoP in several ways: it can
1967 directly repress expression of the *phoRP* regulon, compete for activation of *afsS* or as
1968 activator of *glnR* expression can upregulate expression of the genes responsible for
1969 nitrogen assimilation.

1970
1971 **Figure 2. CCR and the control of antibiotic production.** Glucose repression is shown
1972 for primary and secondary metabolism. Black arrows indicate activation, red lines
1973 repression. Glucose kinase (Glc) is activated post-translationally in a glucose transport-
1974 dependent manner (van Wezel et al., 2007). Glc, glucose; Fru, fructose, secondary sugars
1975 (energetically less favorable sugars, such as lactose, mannitol and glycerol). SI, substrate
1976 induction. Note that glucose is transported by an MFS transporter and not by the PTS in
1977 *Streptomyces*.

1978
1979 **Figure 3. The DasR regulatory network.** The primary metabolism of *S. coelicolor* is
1980 shown for N-acetylglucosamine (GlcNAc), glucose (Glc) and glucosamine (GlcN).
1981 Glucosamine 6- phosphate (GlcN-6P) is a central metabolite that stands at the crossroads
1982 of aminosugar metabolism, glycolysis, nitrogen metabolism and cell wall synthesis. GlcN-
1983 6P and GlcNAc-6P are ligands that modulate the DNA-binding activity of DasR. DasR is a
1984 global repressor of specialised metabolism. Internalised glucose is phosphorylated by
1985 glucose kinase (Glc), which is key to carbon catabolite repression in *S. coelicolor*. In turn,
1986 DasR suppresses CCR by downregulating Glc expression. The broken lines represent
1987 known routes that have not yet been fully characterised.

1988
1989
1990 **Figure 4. Schematic illustration of a selection of genes corresponding to sites of**
1991 **AtrA binding in *S. coelicolor*.** Black and red solid black lines with arrow heads represent

1992 previously described interactions associated with activation by AtrA and repression by
 1993 DasR, respectively. The broken lines represent interactions identified by chromatin
 1994 immunoprecipitation but not yet characterized AtrA binds to upstream regions of genes
 1995 encoding CSRs (*actII-ORF4*, *cdaR* of *S. coelicolor* and *salO* of *S. albus*; the latter encodes
 1996 the CSR for salinomycin biosynthesis). The activator AtrA and the repressor DasR
 1997 compete for binding to the upstream regions of *actII-ORF4* and *cdaR* and upstream
 1998 regions of genes that are involved in the uptake of GlcNAc (*crr* and *nagE2*). In addition
 1999 AtrA binds to an upstream region of SCO0481, which encodes a protein that binds chitin, a
 2000 rich source of GlcNAc. The positive control of AtrA on GlcNAc uptake suggest that AtrA
 2001 increases Act production indirectly through enhanced GlcNAc-induced inactivation of
 2002 DasR as well as directly through activation of *actII-ORF4* transcription. AtrA also binds to
 2003 upstream regions of genes involved in glycerol catabolism (*gylR* and *glpk2* (SCO1658)).
 2004 The binding of AtrA to the upstream region of genes involved in DNA replication (*topA*,
 2005 DNA topoisomerase 1, *uvrA*, *dnaQ*) cell division and sporulation (*ssgR* and *ftsK*)
 2006 explains the role of AtrA in the control of morphological development.

2007

2008 **Figure 5. Initiation of development and antibiotic production.** The developmental
 2009 programme starts with nutrient stress and growth cessation, followed by the accumulation
 2010 of ppGpp. The autolytic dismantling of the cell wall (PCD) releases cell wall-derived
 2011 metabolites that inhibit the activity of the nutrient sensory DasR. The onset of antibiotic
 2012 production correlates temporally to the transition from vegetative to aerial growth, and is
 2013 controlled by multiple pathway-specific and global regulators. Shown here are three key
 2014 pleiotropic regulators, namely the antibiotic repressor DasR which responds to
 2015 phosphorylated aminosugars likely derived from PCD, the activator AtrA (signal unknown)
 2016 and AdpA, which responds to the accumulation of A-factor (synthesized by AfsA). Bld
 2017 proteins and environmental signals control the procession towards aerial growth and
 2018 antibiotic production. Whi proteins control aerial growth. Eventually, FtsZ accumulates and
 2019 localizes to septum sites in an SsgAB-dependent manner. Solid black arrows represent
 2020 major transitions in development. The arrow indicates the FtsZ accumulation checkpoint
 2021 controlled by the Whi proteins. Red lines indicate repression.

2022

2023 **Figure 6. Regulation of glycopeptide biosynthetic gene clusters.** Shown are the
 2024 BGCs for teicoplanin (*tei*), balhimycin (*bal*) and A40926 (*dbv*). Known and putative binding
 2025 sites for StrR (purple) are indicated in the clusters with closed and open circles,
 2026 respectively. The consensus sequence for the StrR binding sites GTCCAR(N)17TTGGAC

2027 is shared between all three BGCs. Genes regulated by LuxR (magenta) are indicated with
2028 an asterisk. Experimentally confirmed operons are indicated with an arrow. The primary
2029 CSR of the teicoplanin BGC is Tei15*, which positively regulates the expression of LuxR-
2030 family regulator Tei16* and of the SARP-family regulator Tei31*, with both regulators
2031 having unknown targets. The *bal* cluster is regulated by the CSR BbR, and lacks a gene
2032 for a LuxR regulator. The primary CSR of the *dbv* cluster is the LuxR regulator Dbv3,
2033 which positively regulates the expression of StrR regulator Dbv4, most likely indirectly. For
2034 details see the text. BGCs adapted from the MiBIG database ⁴¹².

2035

2036 **Figure 7. The regulation of microbisporicin production by *Microbispora corallina*.**

2037 Nutritional stress leads to the RelA-dependent production of ppGpp which results in the
2038 expression of the LuxR-family regulator MibR. MibR activates the expression of
2039 *mibABCDTUV*, which results in the production of an immature and less active form of
2040 microbisporicin (grey circle) and the means for its export. A basal level of expression of the
2041 genes encoding an ECF σ -factor (MibX) / anti- σ -factor (MibW) system enables a feed-
2042 forward regulatory mechanism. The immature compound itself or possibly interaction with
2043 its lipid II to be sensed by MibW, at which point the ECF σ -factor, MibX is released. MibX
2044 then in turn activates its own expression and that of *mibR* as well as the remaining genes
2045 in the BGC.

2046

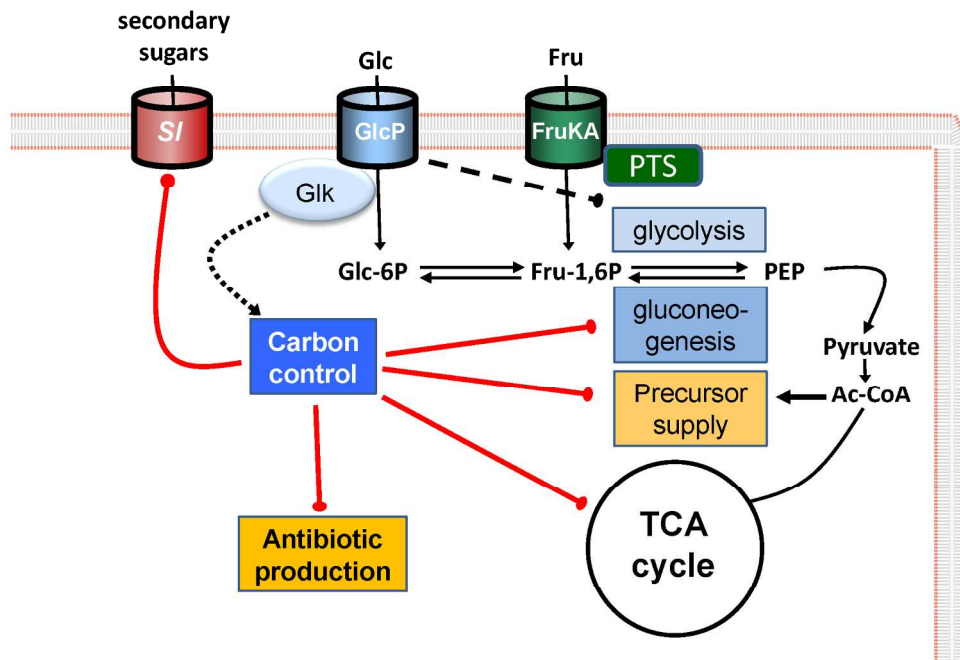


Figure 2

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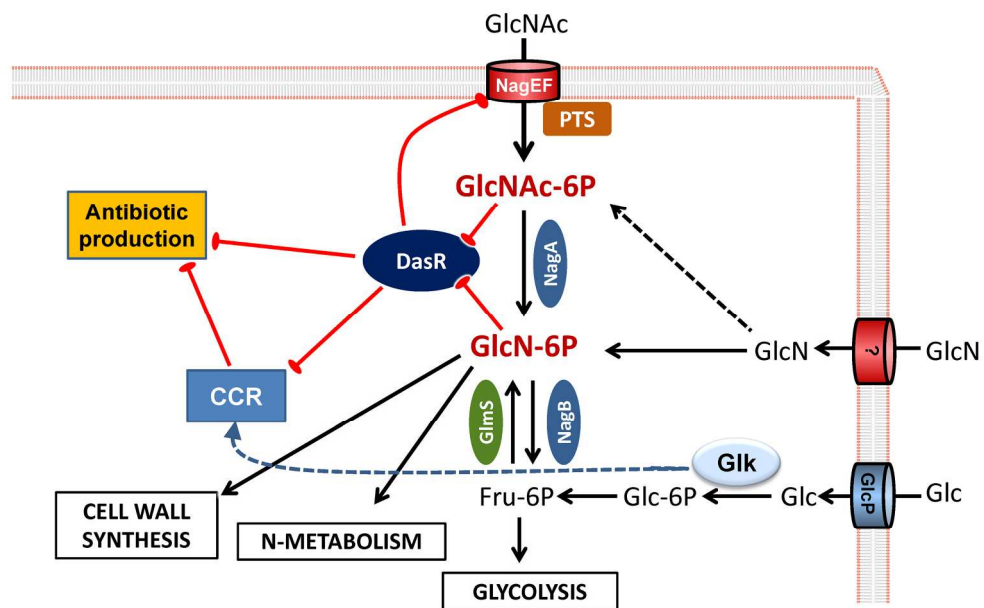


Figure 3

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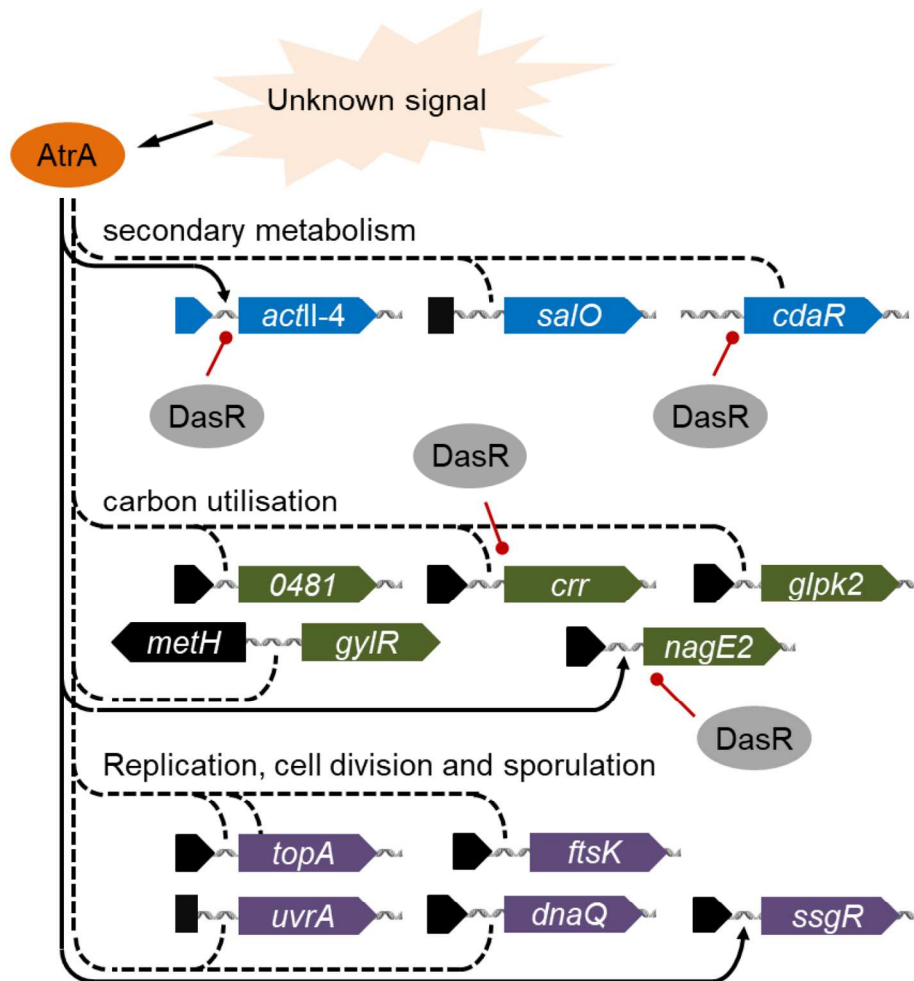


Figure 4

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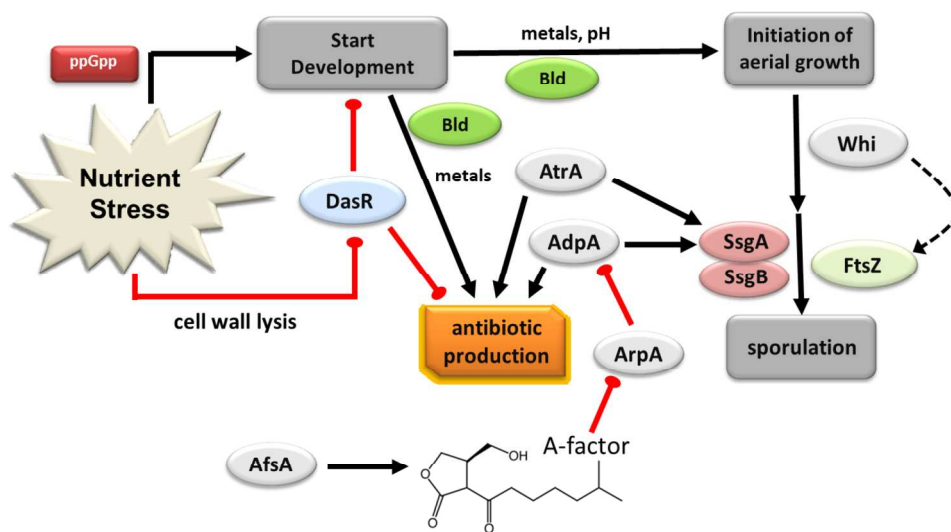


Figure 5

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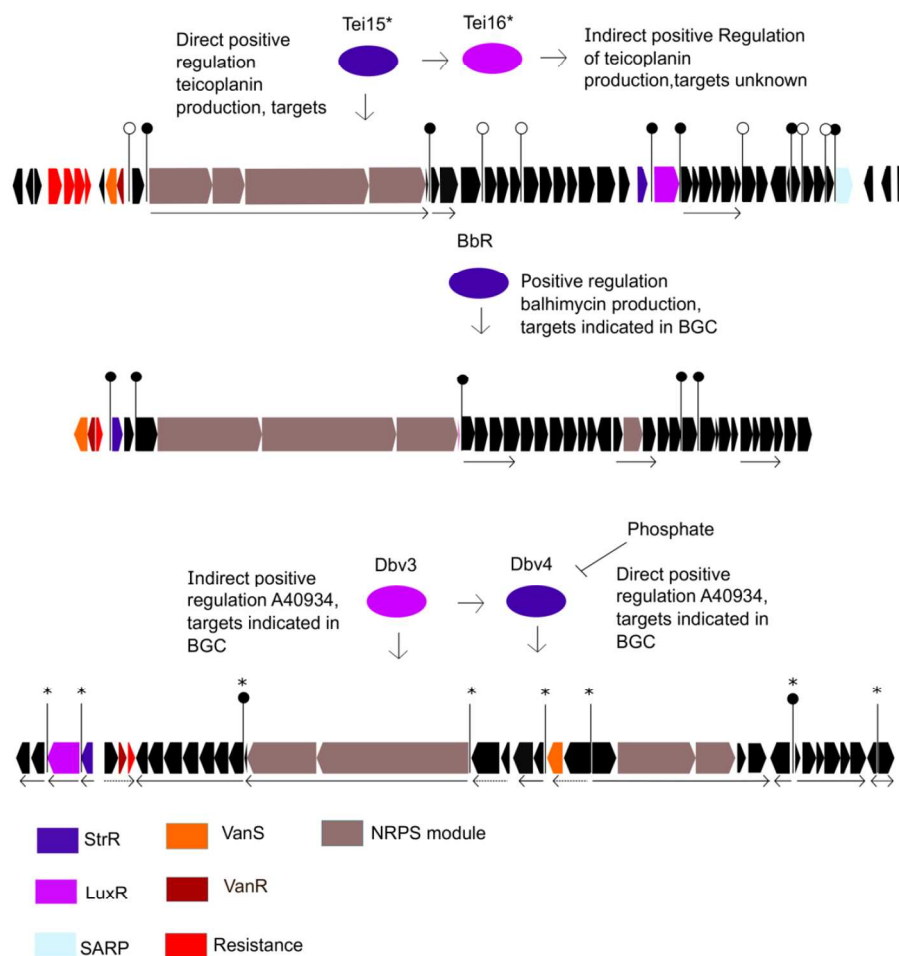


Figure 6

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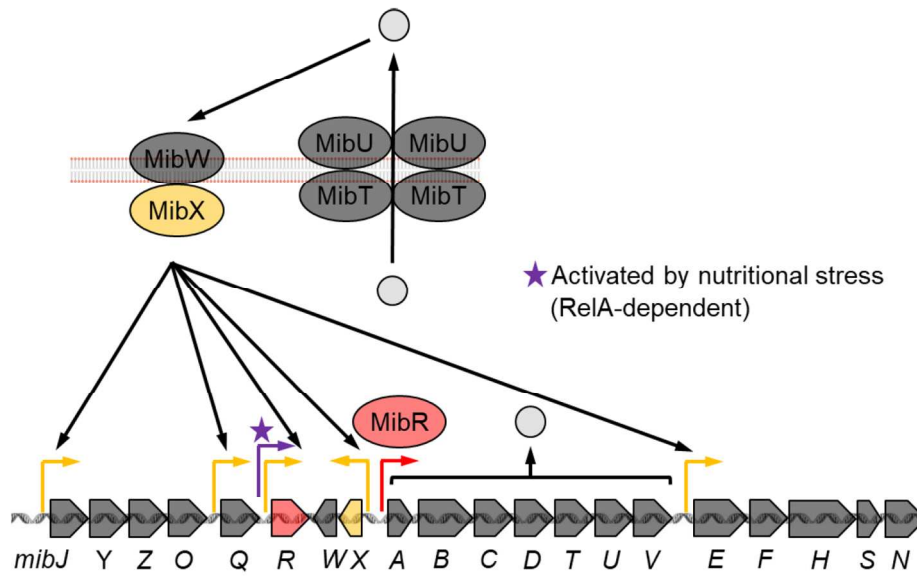


Figure 7

135x84mm (300 x 300 DPI)