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

Helga U. van der Heul,[a] Bohdan L. Bilyk,[b] Kenneth J. McDowall,[b] Ryan F. Seipke[b] and Gilles P. van Wezel[*a]

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

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[a]*Leiden University, Leiden Institute of Chemistry, 2300 RA Leiden, Netherlands. E-mail: g.wezel@biology.leidenuniv.nl*

[b]*Faculty of Biological Sciences, Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT, UK*

1. Introduction

Streptomyces species are renowned for their ability to produce a multitude of bioactive secondary metabolites, some of which have been co-opted clinically as a source of antibacterial, anticancer, antifungal, antiparasitic and immunosuppressive agents.[1–5] The secondary metabolites produced by this taxon offer a chemical diversity that greatly exceeds that of libraries of compounds synthesized chemically and have been pre-selected through millions of years of evolution to interact effectively with biological targets. With the development of numerous approaches for counter selecting compounds with activities that have been previously characterised and in the case of antibiotics might have been rendered ineffective by the emergence of resistance, natural products are being revisited as a potential source of new pharmaceuticals.[6,7]

The biological role of antibiotics has been a topic of some debate. Whilst antibiotics in the natural habitat are typically regarded as weapons, in the same way as they are used in the clinic,[8–10] it has been argued that at least some could function primarily in cell communication and signalling.[11–13] The latter view was based largely on the believe that compounds with antibiotic activity are unlikely to reach concentrations in the soil that block growth, as defined by the minimal inhibitory concentration (MIC). However, selection for resistance occurs even at concentrations far below the MIC and antibiotic-sensitive strains are demonstrably disadvantaged in competing for growth.[14–16]

The majority of the antibiotics that are used in the clinic are produced by Actinobacteria, which are high G + C, Gram-positive bacteria. Of the Actinobacteria, perhaps the most prolific antibiotic producers are members of the genus *Streptomyces*, which belong to the family *Streptomycetaceae*.[2,17,18]

Helga van der Heul, MSc, received her BSc from the Rotterdam University of Applied Sciences, The Netherlands, in 2012 and her MSc in Biology from Leiden University, The Netherlands, in 2014. In the same year she started a position as researcher in the Molecular Biotechnology group led by Prof. Gilles van Wezel. Currently she is a 3rd year PhD student in the same lab, working on the regulation and biosynthesis of natural products in Streptomyces.

Kenneth J. McDowall is a member of the Astbury Centre for Structural Molecular Biology at the University of Leeds. He graduated with a B.Sc. (Hons) in microbiology from the University of Edinburgh (1987); undertook graduate research in the laboratory of Prof. Iain S. Hunter (then at the University of Glasgow), exploring the regulation of oxytetracycline production in S. rimosus and obtaining a Ph.D. in genetics in 1991; and was a postdoctoral researcher in the laboratory of Prof. Stanley N. Cohen at Stanford University, where his interests expanded to include post-transcriptional gene control. In 1996, he was awarded a Royal Society University Research Fellowship and returned to the UK. His current research employs a blend on molecular and 'omics approaches to understand and manipulate gene expression in a range of model bacterial species. He also has a strong interest in student education and currently manages this area for the Faculty of Biological Sciences.

Bohdan Bilyk, PhD, received his BSc and MSc in Biochemistry from Lviv University in 2008. He then moved to the University of Freiburg, Germany, where he held a DAAD Fellowship for one year. In 2010, he moved to the Helmholtz Institute of Pharmaceutical Research Saarland to begin his PhD thesis under the supervision of Prof. Andriy Luzhetskyy, which he finished in 2014. In the same year he started to work as a research associate at PharmBioTec GmbH on a joint project with Sanofi. In 2016, he joined Dr Seipke's laboratory in the Faculty of Biological Sciences at the University of Leeds. He is currently studying the regulation and biosynthesis of microbial natural products.

Ryan Seipke, PhD, was awarded his BA in biology from Adrian College in 2003 and his PhD in microbiology from Cornell University in 2008. He then moved to the University of East Anglia to conduct postdoctoral research and in 2013 was appointed to his current post of Assistant Professor in Bacteriology in the Faculty of Biological Sciences at the University of Leeds. His research interests focus on the regulation, biosynthesis and bioengineering of microbial natural products.

Streptomycetes are found in environments with varying nutrient supply, and metabolise a variety of carbon, nitrogen and phosphate sources. To respond appropriately to the challenges imposed by the environment, the genome of the model streptomycete *S. coelicolor* harbours a staggering 700 regulatory genes.[19] Streptomycetes have a multicellular life cycle, which culminates in sporulation. The reader is referred elsewhere for details of this process.[20–24] In brief, streptomycetes grow as non-motile, vegetative hyphae to produce a network of interwoven filaments called vegetative mycelium. When reproduction is required, for example at the time when nutrients run out, the vegetative mycelium acts as a substrate for newly formed aerial hyphae that eventually differentiate into chains of unigenomic exospores.

Genes required for the transition from vegetative to aerial growth are typically referred to as *bld* genes, referring to their bald phenotype, due to their failure to produce the fluffy white aerial hyphae.[25] Mutants that produce aerial hyphae but no spores are referred to as *whi* mutants, for their white phenotype caused by the lack of the grey spore pigment.[26] Many of the *bld* and *whi* mutants that had been isolated in the 1970s by phenotypic screening have later been identified by genetic complementation experiments, and they have been instrumental in providing better insights into the regulatory cascades that control morphological differentiation. For details we refer the reader to excellent reviews elsewhere.[2,23,27–30]

Production of bioactive compounds is typically linked to the developmental lifecycle, and antibiotics are presumably produced to safeguard the nutrient supply during developmental growth.[31–33] Streptomycetes produce an arsenal of degradative enzymes (*e.g.* glycosyl hydrolases, lipases and proteases), which combined with the production of antibiotics and the ability to form desiccation-resistant exospores has facilitated their success in a multitude of soil environments and sediments including those of marine and freshwater ecosystems. The competitive attributes possessed by streptomycetes have not gone unutilised by higher organisms. For instance, it has become clear that many insects, animals and plants engage in protective symbioses with antibiotic-producing *Streptomyces* species (reviewed in ref. 34 and 35). However, not all interactions between streptomycetes and higher organisms are beneficial – a minority of species produce a cellulose synthase inhibitor called thaxtomin and a coronafacic acid-like phytotoxin, which lead to the development of scab diseases on potato and other tap-root crops.[36,37]

Over the past 50 years, *S. coelicolor* has been the major model for the study of antibiotic production and its control. Early on it was apparent that this strain produced numerous natural products, including actinorhodin (Act[38]), undecylprodigiosin (Red[39]), the calcium-dependent antibiotic (Cda[40]) and plasmid-encoded methylenomycin (Mmy[41]). The genes that encode the machinery for the production of these respective antibiotics are clustered together in 'biosynthetic gene clusters' (BGCs), which typically also harbour resistance gene(s) and one or more transcriptional regulators that control biosynthesis. Sequencing of the *S. coelicolor* genome was a landmark event that revealed an unexpected potential for the production of hitherto unidentified or cryptic natural products,[19] with more than 20 BGCs specifying a diverse range of secondary metabolites.[42,43] One of these is a so-called cryptic polyketide antibiotic (later named coelimycin), which is only produced under specific growth conditions.[44,45] Sequencing of other model Actinobacteria revealed a similar picture, with some species harbouring more than 50 different BGCs.[46–51] Thus, the potential of Actinobacteria as producers of bioactive molecules was found to be much greater than was initially thought. This prompted the sequencing and analysis of the genomes of a large array of species to identify novel BGCs (reviewed in ref. 52–55) plus the development of approaches to induce the production of natural products under laboratory conditions.[1,56–59] The identification of BGCs is now relatively routine using bioinformatics tools, such as antiSMASH,[60] CLUSEAN[61] and PRISM.[62] Available also are tools for the identification of BGCs corresponding to specific classes of natural product, *e.g.* NRPSPredictor for nonribosomal peptides,[63] BAGEL for bacteriocins and lantibiotics[64] and SEARCHPKS for polyketides.[65] For a comprehensive overview of the available bioinformatic tools for genome mining we refer the reader to excellent reviews elsewhere.[66,67]

This review is intended to be an update to our comprehensive review on the same subject published in this journal in 2011.[33] The broad subject is covered, but in the interest of limiting duplicated content, the reader is often referred to our previous review. Here, the focus lies on recent insights into the regulation of natural product biosynthesis in streptomycetes, based on the literature from the period of 2011–2017. The article focuses on both pleotropic and cluster-situated regulators, highlighting recent discoveries. We thereby give specific attention to the control of antibiotic production in other Actinobacteria. We also provide an update on our understanding of the links between primary and secondary metabolism and

Gilles van Wezel is Professor of Molecular Biotechnology at the Institute of Biology, Leiden University. He is also distinguished professor at the Netherlands Ecology Institute (NIOO-KNAW) in Wageningen. He studied Chemistry at the Free University in Amsterdam and obtained his PhD in the lab of Prof. Leendert Bosch at Leiden University in 1994, on the control of protein biosynthesis. He did his postdoc at the John Innes Centre in Norwich with Prof. Mervyn Bibb, on the control of sugar metabolism. He obtained a fellowship from the Netherlands Royal Academy for Arts and Sciences (KNAW) in 2000 and co-founded the Biotech company Mycobics BV in 2001. He obtained a full professorship in 2010. In 2011 his lab developed the practical Antibiotica Gezocht! (Wanted: Antibiotics!), to allow high school pupils to identify antibiotics produced by Actinobacteria in the classroom. His current research focuses on the biology of Actinobacteria and on approaches to discover novel antibiotics and anticancer compounds that should find their way to the clinic.

ecological triggers that stimulate natural product biosynthesis, and outline methodology that could be used to activate silent or cryptic natural product biosynthetic pathways.

2. Transcriptional regulation by cluster-situated regulators

Over the last several decades, investigations into the regulation of the antibiotics produced by *S. coelicolor* (Act, Red, Cda, Mmy and coelimycin) and that of streptomycin biosynthesis by *S. griseus* have established key aspects of the regulation of secondary metabolism in *Streptomyces*. For details we refer to reviews elsewhere.[31–33] The regulation of secondary metabolism is complex and frequently involves pleotropic global regulators that either directly activate or repress biosynthetic genes or do so *via* cluster-situated repressors or activators. A plethora of regulatory proteins is involved in the control of antibiotic production, across a broad range of regulator families. And cross-regulation results in a highly complex regulatory network. This is necessary to correctly interpret the environmental signals and translate them into appropriate transcriptional responses, so as to time the production of natural products, often closely connect to development. The different families of transcriptional regulators known to be involved in the control of antibiotic production, and some well-studied examples, are provided in Table 1.

The regulation of the BGCs for actinorhodin (Act; controlled by ActII-ORF4), undecylprodigiosin (Red, controlled by RedD) and calcium-dependent antibiotic (Cda, controlled by CdaR) of *S. coelicolor* and for streptomycin (Str, controlled by StrR) are the most well-studied examples of cluster-situated regulators (CSRs). ActII-ORF4, CdaR and RedD belong to the SARP family of *Streptomyces* antibiotic regulatory proteins,[68] while StrR unusually belongs to the family of ParB-Spo0J proteins, most of which are involved in DNA segregation and sporulation.[69] All available evidence supports the conclusion that the cellular level of a cluster-situated regulator dictates the level of transcription of its cognate BGC, which correlates closely with the level of production of the corresponding natural product.[70,71] Indeed, the timing of Red production fully depends on the promoter that drives the transcription of *redD*, allowing its use as a transcriptional reporter system.[72] Thus, the ultimate factor deciding whether or not a BGC is expressed is its CSR(s). While ActII-ORF4 and StrR act as single CSRs within their respective BGCs, production of RedD is in turn controlled by RedZ,[73,74] which is related to the response regulators (RR) of prokaryotic two-component systems (TCS) but 'orphaned', *i.e.* not genetically linked to a histidine kinase.[75] It is becoming increasingly clear that the presence of multiple CSRs is more often the rule than the exception with each regulator effecting control of a subsets of genes or contributing to a hierarchical cascade. The latter is exemplified by the BGCs specifying polyene antifungal compounds such as amphotericin, nystatin, natamycin (pimaricin) and candicidin.[76–79] It has been assumed and, in some cases, shown that many regulators are responsive to small molecule signals. Regulators responsive to autoregulatory molecules such as γ-butyrolactones are well known,[80,81] and feedback control by biosynthetic intermediates over production or export has been demonstrated for jadomycin, Act and simocyclinone biosynthesis.[82–84] However, the identity of the ligands/signals perceived by both pleotropic and CSRs is a major question within the field, and if answered could lead to a revolution in chemical genetic tools for the stimulation of natural product biosynthesis, and thus drug discovery.

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

The first complete regulatory pathway leading to activation of a BGC was described for Str in *S. griseus*.[85] Transcription of StrR, which as mentioned above is the corresponding CSR, is activated by the pleiotropic regulator AdpA (A-factor-dependent protein);[86] whose transcription depends on the accumulation of the γ-butyrolactone 2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone, better known as A-factor. The hormone-like compound binds to ArpA,[87] which acts as a repressor of *adpA* transcription.[88] AdpA also activates morphological differentiation, and thus plays a key role in the coordination of chemical and morphological differentiation.[89,90] A-Factor is synthesized by the enzyme AfsA.[91] The role of A-factor in the control of antibiotic biosynthesis is further discussed in Section 9.

The transcription of *strR* is subject to multi-level control, and in particular by the pleiotropic regulator AtrA,[92,93] which has an orthologue in *S. coelicolor* that activates transcription of *act*II-ORF4, the CSR within the *act* cluster.[94] Binding of AtrA *in vivo* within the vicinity of the *act*II-ORF4 promoter has recently been confirmed by chromatin immunoprecipitation in combination with DNA sequencing (ChIP-seq) (McDowall *et al.*, unpubl. data). Compared to what is known about *strR*, the control of *act*II-ORF4 is complex with many transcription factors reported to control its expression directly. Numerous direct and indirect regulators have been identified.[32,33] Some of the most recent examples are summarized in Table 2. For some of these transcription factors, binding has been demonstrated *in vivo* by ChIP-based approaches. In addition to AtrA, these include DasR,[95] a member of the GntR family that controls the uptake and metabolism of *N*-acetylglucosamine (GlcNAc) and the degradation of chitin to GlcNAc,[96,97] AbsA2,[98] the response regulator of the AbsA TCS, which negatively controls antibiotic production in *S. coelicolor*,[99,100] AbrC3,[101] a response regulator of a TCS that is atypical in having two histidine kinases,[102] and Crp,[103] the cyclic AMP receptor protein, which is perhaps best known for mediating carbon catabolite repression of the *lac* operon in *E. coli*,[104] controls diverse cellular processes in many bacteria,[105] and is a key regulator of secondary metabolism as well as spore germination and colony development in *S. coelicolor*.[106] In addition to direct regulation, the expression of *act*II-ORF4 is dependent on *relA*,[107] which is required for induction of the stringent response. The stringent response enables bacteria to survive sustained periods of nutrient deprivation by enhancing the transcription of numerous genes required to survive stress, while lessening transcription of genes, such as those specifying stable RNAs, whose products are required in significantly reduced amounts during periods of slowed

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

Family[d]	Example	Host[a]	Control[b]	Target BGC[c], comment	Reference
SARP	ActII-ORF4, RedD, CdaR	*S. coelicolor*	(+)	Act, Red, Cda, respectively	68
	AfsR	*S. coelicolor*	(+)	Activates transcription of AfsS	138
	FarR3/Far4	*S. lavendulae*	(+, −)	Indigoidine, nucleoside and D-cycloserine	299
StrR (ParB-Spo0J)	StrR	*S. griseus*	(+)	Streptomycin	391
	Tei15*	*Actinoplanes teichomyceticus*	(+)	Teicoplanin	332 and 335
	Dbv4	*Nonomuraea* sp. ATCC39727	(+)	A40926	330 and 336
LAL	FscRI	*S. albus*	(+)	Candicidin and antimycin	121
	AveR	*S. avermitilis*	(+, −)	Avermectin and oligomycin	392
	Dbv3	*Nonomuraea* sp. ATCC39727	(+)	A40926	330
TetR	AtrA	*S. griseus*	(+)	Global regulator	92
	ArpA	*S. griseus*	(−)	GBL receptor, repressor of *adpA*	86
	ScbR	*S. coelicolor*	(+, −)	GBL receptor	143
AraC/XylS	AdpA	*S. griseus*		Activates StrR expression	90
GntR	DasR	*S. coelicolor*	(+, −)	Global regulator of antibiotic production; effector molecule is *N*-acetylglucosamine	95 and 194
c-AMP receptor protein	Crp	*S. coelicolor*	(+)	Regulator coordinating development, primary and secondary metabolism	103
Orphan RR	RedZ	*S. coelicolor*	(+)	Red	73
	GlnR	*S. coelicolor*	(+)	Act and Red	145 and 151
TCS	AbsA1/AbsA2	*S. coelicolor*	(−)	Act, Red, Cda	98
	AfsQ1/2	*S. coelicolor*	(+)	Act, Red, Cda; responds to nitrogen	111
	PhoRP	*S. coelicolor*	(+, −)	Act; global regulator	129 and 230
	DraR/K	*S. coelicolor*	(+, −)	Act, Red, coelimycin, responds to high concentrations of nitrogen	113
	OsdR/K	*S. coelicolor*	(+)	Act, responds to oxygen level	112
ROK	Rok7B7	*S. coelicolor*	(+, −)	Act, Red, Cda; CCR	209 and 210
σ Factor	MibX/MibW	*Microbispora corallina*	(+)	Microbisporicin	350 and 352
	AntA	*S. albus*	(+)	Antimycin	359
BldB	BldB	*S. coelicolor*	(+)	Antibiotic production, development and CCR	187–189
tRNA	BldA	*Streptomyces* species		Leucine-tRNA for UAA codon. Translational control of antibiotic production and morphogenesis	393
XRE	MmyB	*S. coelicolor*	(+)	Methylenomycin B; controlled by furans	286 and 394
Wbl (WhiB-like protein)	WblA	*S. coelicolor*	(−)	Pleiotropic regulator of antibiotic production and development	395
LacI	AcrC	*Actinoplanes* sp. SE50/110	(−)	Acarbose	396
LmbU		*S. lincolnensis*	(+, −)	Lincomycin	397 and 398
Lrp/AsnC	SCO3361	*S. coelicolor*	(+)	Act; control by amino acids	399
NsdA	NsdA	*S. coelicolor*	(−)	Act, Cda, Mmy	400
IclR	NdgR	*S. coelicolor*	−	Act; dependent on amino acids	401
MarR	DptR3	*S. roseosporus*	+	Daptomycin	402

[a] *Streptomyces* abbreviated with '*S.*'. [b] Activation indicated by +, repression by −. [c] Act, actinorhodin; Cda, calcium-dependent antibiotic; Red, prodiginines; Mmy, methylenomycin. [d] LAL, large ATP-binding regulators of the LuxR family (in the text mentioned as LuxR); XRE, xenobiotic response element.

growth.[108,109] Whilst the signals transduced by Crp and the stringent response are well described, the signals sensed or transduced by most of the transcription factors that bind the *act*II-ORF4 promoter remain to be elucidated. An exception is DasR, which is a receptor for glucosamine-6-phosphate (GlcN-6P), an intermediate in GlcNAc metabolism, and derivatives.[97] The binding of GlcN-6P by DasR reduces its affinity for DNA, which de-represses the expression of genes that facilitate the degradation of chitin to GlcNAc and its uptake and metabolism.[96,97] Links between DasR and AtrA are described later in this review (Section 5.3).

In addition to AraC and AbsA, several other TCSs regulate secondary metabolism in *S. coelicolor* and other Actinobacteria.[110–113] TCSs are the major signal-transduction systems of bacteria and enable them to monitor and adapt to environmental changes.[114,115] Streptomycetes harbour a large number of TCSs, which likely reflects the changing and variable nature of their natural habitats.[19,110,116] The PhoRP TCS system is ubiquitous in bacteria and senses phosphate and regulates its assimilation. PhoRP plays a major role in the control of antibiotic production in streptomycetes.[117–119] Similar has been found for the AfsQ1/2 TCS, which controls the biosynthesis of Act, Red and Cda in response to nitrogen limitation[111] *via* what appears to be direct interaction with the promoter regions of *act*II-ORF4, *redZ* (which activates *redD*) and *cdaR*, respectively. The AfsQ1/2 TCS is closely related to CseBC, which responds to

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[a]	Function(s) of the regulator(s)[b]	Ref.
Regulators known to directly control antibiotic BGCs			
mtrAB	SCO3013/2; SVEN2756/5	TCS; MtrA activates *act*II-ORF4 and *redZ* and links their production to development	403
draRK	SCO3063/2; SAV3481/0	TCS; regulator of *act*II-ORF4 and *kasO* in *S. coelicolor* and of *olmRI* in *S. avermitilis*. Impacts Red and ave production in *S. coelicolor* and *S. avermitilis*, resp	113
	SCO3361	Lrp/AsnC family positive regulator for Act production. Binds to *act*II-ORF4 (EMSA)	399
crp	SCO3571	Regulator of primary and secondary metabolism; activates *act*II-ORF4, *cdaR* and *cpkA* (Chip-seq)	103
glnR	SCO4159; SAV4042	Activator of *act*II-ORF4 and repressor of *redZ* in *S. coelicolor* (EMSA). Activator of *aveR* (avermectin) and repressor of *olmRI*/*olmRII* (oligomycin) in *S. avermitilis* (EMSA)	152
abrC1C2C3	SCO4596	Atypical TCS with two kinase (C1 and C2); response regulator AbrC3 is a transcriptional activator of *act*II-ORF4 (ChIP-chip); impacts Red production	101
lexA	SCO5803	Global regulator of the DNA damage response; repressor of *act*II-ORF4 (EMSA)	404
	SCO6256	GntR family regulator of antibiotic production. Direct activator of *cdaR* and indirect repressor of Act production (EMSA)	405
scbR2	SCO6286	Activator of *act*II-ORF4, *redD*, *redZ* and *cdaR*, repressor of *cpkO* and SCO6268 (*cpk* cluster) (Chip-seq, EMSA)	143
Regulators in pathway with missing link to antibiotic gene clusters			
ohkA	SCO1596; SAV6741	Orphan HK; plays global role in antibiotic biosynthesis, by influencing precursor supply, pleiotropic and pathway-specific antibiotic regulators	406
abrA1A2	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
aor1	SCO2281	Orphan response regulator; upregulates Act, Red and Cda production and downregulates *sigB*, thus linking antibiotic production to osmotic stress response	408
stgR	SCO2964	LTTR; negative regulator for Act and Red production trough upregulation of *act*II-ORF4 and *redZ*, respectively. Exact regulatory cascade remains unknown	409
sigT	SCO3892	ECF sigma factor; required for normal Act production under nitrogen limitation	358
cmdABCDEF	SCO4126 –SCO4131	Operon for membrane proteins; affects differentiation and causes increased production of Act	410
phoU	SCO4228	Activates Act and Red production. Exact regulatory cascade unknown	411

[a] SCO, *S. coelicolor*; SAV, *S. avermitilis*; SVEN, *S. venezuelae*; see StrepDB for the full annotation (http://strepdb.streptomyces.org.uk). [b] Experimental evidence presented between brackets (EMSA, Electromobility shift assay; ChIP-Seq, chromosome immunoprecipitation combined with next-generation sequencing).

cell-envelope stress.[75] Recently, it was shown that the DraRK TCS, which responds to high concentrations of nitrogen,[113] and the OsdRK TCS, which is oxygen-responsive, are similar in function to the system controlling dormancy in mycobacteria,[112,120] and are both required for Act production. Interestingly, in the absence of a functional DraRK system the production of Cpk and Red increases.[113] The AbsA system has been exploited to improve the chance of success during screening of streptomycetes for new antibiotics by overexpression of the *S. coelicolor* homologue in other streptomycetes; this led among others to the induction of pulvomycin production in *S. flavopersicus*. Cross-talk between the different regulatory networks is discussed in Sections 5 and 6.

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

It is well established that a CSR usually binds to promoter sequence(s) and either activates or represses genes only within its cognate BGC. For examples see Tables 1 and 2. However, this is not strictly true for all CSRs. Recently, the PAS-LuxR family cluster-situated regulator within the candicidin BGC was shown to not only activate 16 out of the 21 genes in the gene cluster, but also to be required for expression of the antimycin BGC.[79,121] Thus, antimycin and candicidin biosynthesis are co-ordinately controlled by FscRI in *S. albus*.[121] A similar observation was made in *S. avermitilis*, where PteF, a member of PAS-LuxR family and cluster-situated activator of the filipin BGC, was proposed to cross-regulate the production of oligomycin.[122] Thus, evidence is accumulating, at least for PAS-LuxR family regulators, that they may not in fact simply be CSRs but act more broadly to co-ordinately control the biosynthesis of multiple compounds. This is likely rooted in the flexible inverted repeat the family of regulators appears to bind to both *in vitro* and *in vivo*.[121,123] It is an obvious and attractive hypothesis that production of secondary metabolites with antimicrobial properties or subsets thereof should be coordinated, so as to maximise any synergistic activity and minimise the development of resistance to the agents produced.

3. The impact of phosphate availability on secondary metabolism

The impact of phosphate availability on bacterial physiology and gene expression in particular has been intensely studied in

Streptomyces species and other bacteria.[124–127] Expression of a suite of genes involved in phosphate management termed the *pho* regulon is controlled by the PhoRP TCS.[116,128,129] During phosphate starvation, the membrane-bound sensor kinase, PhoR, undergoes autophosphorylation and transfers its phosphate group to the response regulator, PhoP[119,130] (Fig. 1). The phosphorylated form of PhoP (PhoP-P) binds to a well conserved DNA motif called a PHO box and can either activate or repress expression of genes within the *pho* regulon.[118] During growth in phosphate replete conditions, PhoR is prevented from phosphorylating PhoP *via* physical interaction with the phosphate-specific transport (Pst) system, a high-affinity phosphate transport system whose production is activated by PhoR.[118,131,132] This interaction creates a regulatory loop in which the Pst system is produced at a low level during conditions of phosphate sufficiency. When phosphate levels drop, PhoR is released and phosphorylates PhoP, which then activates transcription of genes within the Pst system and the other genes within the *pho* regulon.[118] The precise signal that frees PhoR to phosphorylate PhoP is unknown, but it is known that the switch is reversible.

It has been known for some 15 years that deletion of *phoP* can lead to earlier and increased production of antibiotics.[119] This phenomenon was covered in our previous review[33] and for *S. coelicolor* was rooted in destabilization of a negative regulatory loop involving the AfsKRS system.[133,134] AfsR is a transcription factor related to SARPs that when phosphorylated by AfsK activates transcription of the gene encoding AfsS, a small sigma factor-like protein required for antibiotic biosynthesis in *S. coelicolor*.[135–138] In the proposed regulatory loop, PhoP represses the production of AfsS and AfsR represses the production of PhoRP and the Pts system.[135] However, recently PhoP was shown to in fact be an activator of *afsS* transcription in experiments using a full panel of *phoP*, *afsR* and *afsR/phoP* mutants and a suite of synthetic promoters engineered to prevent AfsR binding but not PhoP binding.[139] In a revised model, PhoP hinders higher activation of *afsS* transcription by AfsR by outcompeting AfsR for binding to the *afsS* promoter (Fig. 1).[135,139]

A series of ChIP-Chip experiments were conducted with *S. coelicolor*, which provided genome-wide insight into the role of PhoPR in controlling secondary metabolism.[140] These revealed that PhoP serves as a master regulator of secondary metabolism during phosphate starvation, whereby it transiently represses pleotropic activators of antibiotic production and regulators of morphological development, namely *bldA*, which specifies the leucine tRNA corresponding to the rare UUA codon, and *scbAR*, which encodes the γ-butyrolactone regulatory system of *S. coelicolor* that positively influence morphological development, and Act and Red biosynthesis.[141,142] Interestingly, the ScbAR system also indirectly controls the gene expression of *scbR2* whose gene product activates *afsK* expression,[143] which is the cognate sensor kinase responsible for activating the global regulator of secondary metabolism, AfsR (mentioned above). Thus, although PhoP activates expression of *afsS*, it also indirectly represses transcription of *afsK*, which means AfsR remains unphosphorylated and inactive (Fig. 1).

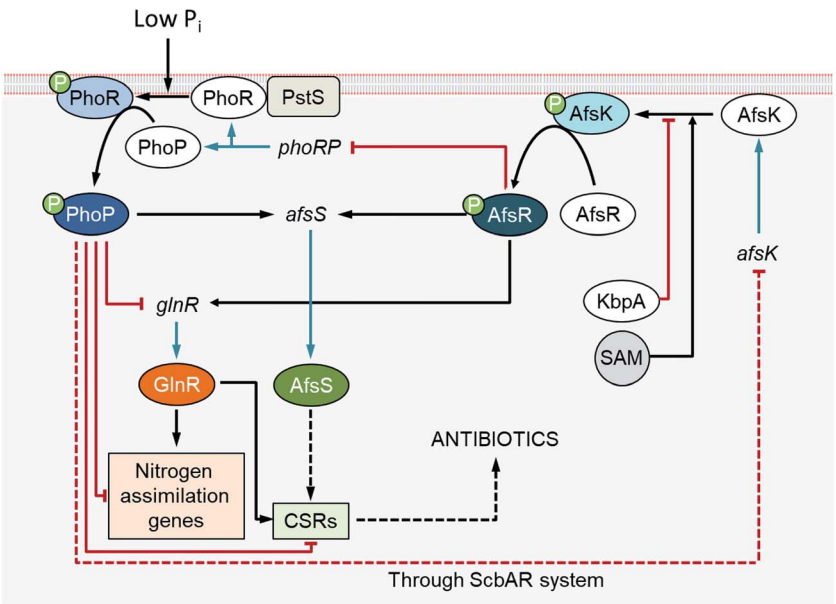

Fig. 1 The PhoRP and AfsKRS systems and their interplay in regulation of nitrogen metabolism and antibiotic production. Black arrows indicate activation and red bars indicate repression, cyan arrows indicate expression of genes. During growth under phosphate deplete conditions, the global regulator PhoP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

Although there are only a handful of example thus far, it is clear that in addition to controlling pleotropic regulators, PhoP can also act directly upon BGCs. For example, in *S. coelicolor*, PhoP negatively regulates the biosynthesis of Cda by repressing the *cdaR* gene.[140] Interestingly, the inverse seems to be the case for the BGC specifying coelimycin where there are three PHO boxes within the DNA sequence of two structural genes and expression of the gene cluster appears to be PhoP-dependent.[140] Direct regulation of biosynthetic pathways by PhoP is not a peculiarity of *S. coelicolor*, as PhoP was recently shown to negatively regulate avermectin biosynthesis by repressing the expression of *aveR*, which encodes a cluster-situated activator.[143]

4. Regulation of secondary metabolism by nitrogen

The uptake and incorporation of nitrogen is essential for anabolism of amino acids, nucleic acids and peptidoglycan, among other important macromolecules. *S. coelicolor* can utilise diverse nitrogen sources including ammonia, nitrate, nitrite, urea, amino sugars and amino acids.[144–146] Assimilation of nitrogen results in the production of glutamate and glutamine, which act as the primary nitrogen donors within the cell.[147] Like other bacteria, *Streptomyces* species possess a sophisticated regulatory system that enables adaptation to nitrogen availability. Many studies have indicated that the source of nitrogen can influence the production of secondary metabolites. The production of most of the secondary metabolites is reduced by nitrogen sources that are favourable for growth.[148,149] This is presumably because utilization of a high-quality nitrogen source (*e.g.* ammonium) causes more of the available carbon to be consumed for growth and generation of biomass and thus ultimately less carbon is available for secondary metabolism when starvation occurs. Although the above has been known for a long time, the underpinning molecular detail has taken longer to elucidate. The global regulator controlling nitrogen metabolism is GlnR, which is an orphan response regulator without a cognate sensor kinase (Fig. 1).[145,150] Deletion of *glnR* in *S. coelicolor* blocks production of Act and Red.[151] GlnR-mediated regulation of Act and Red production was assumed to be indirect until a recent study demonstrated otherwise. *In vitro* DNA binding and DNaseI footprinting studies showed that GlnR binds the promoter sequence of CSRs within these BGCs (*act*II-ORF4 and *redZ*, respectively), implying that GlnR regulation is direct.[152] In the same study, direct regulation of CSRs of avermectin and oligomycin biosynthesis (*aveR* and *olmRI/RII*, respectively) by GlnR in *S. avermitilis* was also demonstrated; thus, direct regulation of a subset of natural product BGCs by GlnR is likely to be universal.[152] Several studies have recently been conducted that have enhanced the understanding of nitrogen metabolism and its interconnectedness with phosphate and carbon utilization. These connections and their implications for secondary metabolism are further discussed in Section 6.

5. Control of antibiotic production by the carbon source

5.1. Carbon catabolite repression and the control of antibiotic production

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

In most bacteria, glucose is transported through the phosphoenolpyruvate-dependent phosphotransferase system or PTS. The PTS encompasses Enzyme I (EI) and phosphocarrier protein HPr in combination with carbohydrate-specific transport complexes called Enzyme II (EII), which confer substrate specificity.[165,166] As a result, the PTS typically plays a key role in glucose repression.[104,167,168] However, in *Streptomyces* species, deletion of either of the genes *ptsH*, *ptsI* or *crr* for HPr, EI and EIIA, respectively, has no influence on CCR, but instead leads to a block in morphological differentiation, with mutants failing to produce aerial hyphae and/or spores on a reference medium such as R2YE agar.[97,169] This sporulation defect is surprising and may be associated with lack of iron and/or copper in this medium, accompanied by a reduced production of the siderophore, desferrioxamine.[170–172] This link between carbon availability, iron homeostasis and morphological differentiation has not yet been resolved. The limited role of the PTS in CCR may be explained by the fact that in streptomycetes, glucose is internalized *via* the GlcP permease, which belongs to the major facilitator subfamily of transporters.[173–175] For a summary of central carbon metabolism and CCR, see Fig. 2.

It was recognized many decades ago that randomly generated mutants lacking CCR are invariably mutated in the gene *glkA*, which encodes a glucose kinase.[176,177] Indeed, a targeted deletion of *glkA* in a clean genetic background was pleiotropically defective for CCR.[178–180] The activity of Glk is mediated by as of yet unknown mechanism.[181] Its role in catabolite repression may be co-ordinately controlled with a number of other proteins. These include SCO2127, a protein of unknown function, which is encoded by the gene upstream of *glkA*[182,183] and regulatory proteins that control the transcriptional network of genes that mediate CCR, such as the global regulators Rok7B7 and DasR (see below). Another interesting protein is the phosphoinositide phosphatase, SblA.[184] Deletion of *sblA* in *Streptomyces lividans* leads to relief of CCR, with accelerated growth and development in the presence of glucose on some media.[185] These phenotypes correlated with reduced glucose uptake by

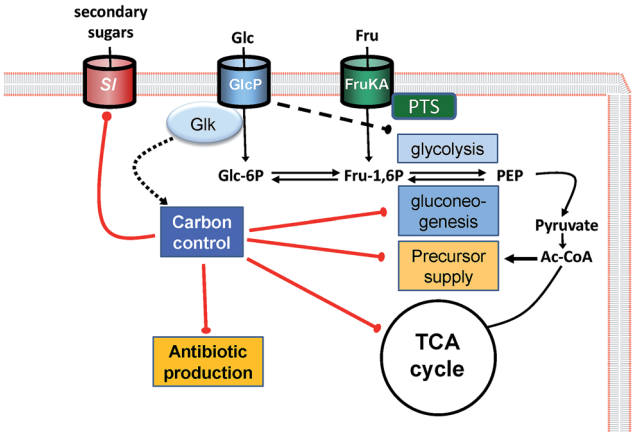

Fig. 2 CCR and the control of antibiotic production. Glucose repression is shown for primary and secondary metabolism. Black arrows indicate activation, red lines repression. Glucose kinase (Glk) is activated post-translationally in a glucose transport-dependent manner (van Wezel *et al.*, 2007). Glc,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

the mutant and may therefore affect the activity of GlcP. The cleavage of phosphoinositides by SblA is apparently required to resume growth in transition phase, although the mechanism has not been elucidated.[185]

Studies with *S. peucetius* suggested the existence of an integral regulatory system that responds to glucose transport and metabolism, which probably elicits CCR.[154] Indeed, addition to growth media of either of the glycolytic intermediates fructose 1,6-biphosphate and phosphoenolpyruvate results in glucose repression of daunorubicin and doxorubicin biosynthesis in *S. peucetius*.[186] This connects to observations that the activity of GlkA depends on interaction with the glucose permease GlcP in *S. coelicolor*.[181]

Many antibiotics show growth phase-dependent control. As a consequence, developmental mutants that are blocked in an early phase of the life cycle – in particular *bld* mutants – typically fail to produce antibiotics. A well-studied case is represented by mutants that lack the developmental gene, *bldB*, as these are not only disturbed in development and antibiotic production, but are also defective in CCR.[187,188] This links the pathways that regulate carbon utilization and morphological differentiation. BldB is a member of a family of DNA-binding proteins that are only found in Actinobacteria. The family is widespread in streptomycetes, with several paralogues in *S. coelicolor*, including AbaA and WhiJ, which play a role in the control of antibiotic synthesis and development, respectively.[189] Identification of the BldB regulon and the way its activity is modulated will likely offer important new insights into the growth phase-dependent control of antibiotic production and the role of CCR in this process.

5.2. New insights into the nutrient-sensory DasR system

In streptomycetes, the PTS plays a major role as the first step in a global antibiotic sensory system revolving around the nutrient sensory protein, DasR, which is conserved in streptomycetes and many other Actinobacteria. DasR is a GntR-family repressor with a pleiotropic role in the regulation of primary and secondary metabolism and of development. For details, we refer to reviews elsewhere.[33,190] Here we summarise the key elements of the regulon and highlight recent insights (Fig. 3). The core regulon of DasR in all Gram-positive bacteria revolves around the genes for aminosugar transport (*pts*) and metabolism (*nag*)

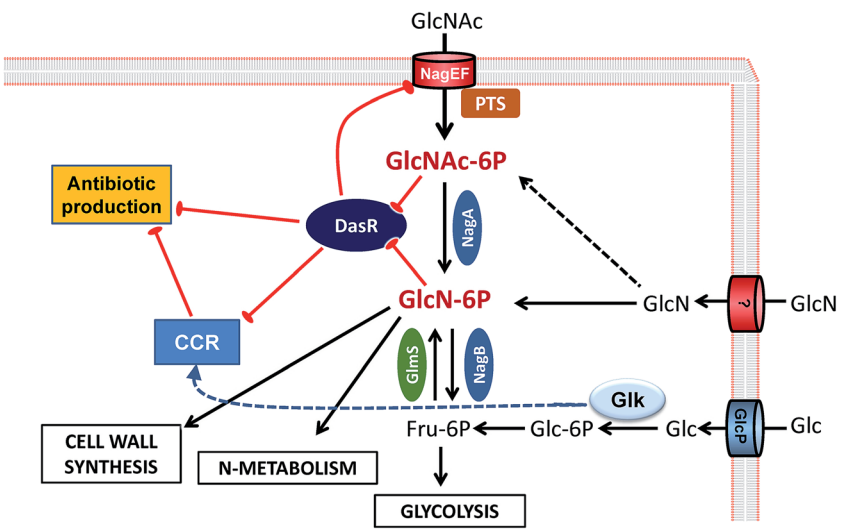

Fig. 3 The DasR regulatory network. The primary metabolism of *S. coelicolor* is shown for *N*-acetylglucosamine (GlcNAc), glucose (Glc) and glucosamine (GlcN). Glucosamine 6-phosphate (GlcN-6P) is a central metabolite that stands at the crossroads of aminosugar metabolism, glycolysis, nitrogen metabolism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

and in streptomycetes also the genes for the chitinolytic system (*chi*). Originally identified as the repressor of the chitobiose transporter DasABC,[191,192] it was soon recognized that DasR also controls many genes involved in antibiotic production. Comprehensive analysis of the DasR regulon of *S. coelicolor* showed that it acts as a direct and very global transcriptional repressor of antibiotic production by binding to the promoter regions of the CSRs for all known chromosomally located antibiotic BGCs in *S. coelicolor*.[95,97,193,194] DasR also represses siderophore biosynthesis *via* control of the iron-homeostasis regulator *dmdR1*.[170,195] A similar pleiotropic role of DasR has also been reported in the erythromycin producer *S. erythraea*,[196,197] but is not typical of all streptomycetes.

The DNA-binding activity of DasR is modulated by ligands derived from GlcNAc or glucosamine (GlcN), in particular GlcNAc-6P and GlcN-6P, and the crystal structure of DasR and its orthologue NagR of *Bacillus subtilis* in complex with these ligands have been elucidated.[198,199] GlcN-6P stands at the crossroads of carbon and nitrogen metabolism and cell-wall synthesis, and by acting as an effector of the DasR-dependent antibiotic control system, it plays a major role in the connection between primary and secondary metabolism (Fig. 3). The DNA-binding activity of DasR depends on environmental conditions. High concentrations of GlcNAc under famine conditions (*e.g.* on minimal media) result in inactivation of DasR, and thus derepression of its targets, leading to enhanced antibiotic production and development. Conversely, on rich media, GlcNAc represses antibiotic and development, leading to a complete developmental block.[97,194,200] This phenomenon is known as *feast* or *famine*; under conditions of nutritional richness, aminosugars are perceived as derived from chitin, signalling plenty of nutrients, while under poor growth conditions (famine) it is perceived as coming from autolytic degradation of the cell wall and hence cell death. The latter elicits development and antibiotic production. Besides the phosphorylated aminosugars GlcN-6P and GlcNAc-6P, other metabolites may also modulate the DNA-binding activity of DasR. These include high concentrations of phosphate (organic or inorganic), which were shown to enhance the binding of DasR to its recognition sites.[95,201] Thus, the affinity of DasR for its recognition sites (and with that the expression of its regulon, including many BGCs for natural products) depends on the metabolic status of the cell. Interestingly, high concentrations of phosphate (either organic or inorganic) enhance binding of DasR to its recognition site *in vitro*, which reinforces the PhoP-mediated repression of antibiotic production by phosphate.[95,201]

Full genome-scale identification of the DasR binding sites *in vivo* using ChIP-chip analysis corroborated the identity of canonical DasR binding sites or *dre* (DasR-responsive elements), but also revealed so-called class II sites, which do not conform to the known consensus sequence.[95] These sites are not found by the regulon prediction algorithm PRE-Detector.[202] Binding of DasR to class II sites may require a co-repressor, which has not yet been identified. The ChIP-Chip analysis also showed that the binding profile of DasR changes dramatically over time, with only small overlap in the binding profiles between 24 (vegetative growth) and 54 hours (morphological differentiation and antibiotic production). Thus, the DasR regulon is a highly complex system, which is influenced by metabolic status and most likely also by other regulatory proteins. Taken together, the metabolic status of the cell determines the selectivity of DasR for its recognition sites and thus the expression of its regulon, which includes many secondary metabolite BGCs.

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

Until the discovery of DasR, it was unclear how global carbon control was related to the control of specific carbon utilization regulons and antibiotic biosynthetic genes. Deletion of the genes for either GylR or MalR relieves both CCR and substrate induction of glycerol and maltose utilization, respectively, and hence gives constitutive expression even in the absence of inducer, while over-expression results in hyperrepression.[203,204] This suggests that a global regulatory system for carbon utilization does not exist in *S. coelicolor*. In most bacteria, global carbon control depends on the cAMP receptor protein (CRP). Streptomycetes do have a cAMP receptor protein, but in contrast to other bacteria, it does not seem to play a role in CCR. Instead, CRP plays a role in the control of germination, and *crp* null mutants show prolongued dormancy.[106] Importantly, genome-wide DNA binding studies and transcriptional analysis revealed that CRP also globally controls antibiotic BGCs in *S. coelicolor* (ref. 103; see also Section 6).

There is also growing evidence that besides DasR, the TetR-family regulator AtrA plays a role in carbon utilization (Fig. 4). Very recent ChIP-seq experiments (McDowall *et al.*, unpubl. data) have confirmed that AtrA binds upstream of *nagE2*, which encodes a known permease for the uptake of GlcNAc.[205] Similar to what was found for *act*II-ORF4, this binding appears to activate transcription as disruption of *atrA* results in reduced levels of *nagE2* transcript (Nothaft *et al.*, 2010). This led to the suggestion that AtrA may increase Act production indirectly through enhanced GlcNAc-induced inactivation of DasR as well as directly through activation of *act*II-ORF4 transcription (Nothaft *et al.*, 2010). The control of DasR activity by AtrA *via* cellular levels of GlcNAc may extend beyond *nagE2* as recent ChIP-seq also identified AtrA binding to recognisable motifs upstream of SCO0481, which encodes a protein that binds chitin (a rich source of GlcNAc), and *crr* (SCO1390), for the global PTS component EIIA, that is required for GlcNAc transport. The role of AtrA in carbon utilisation almost certainly extends beyond GlcNAc metabolism (Fig. 4). ChIP-seq also identified AtrA binding to sites upstream of *gylR* (SCO1658) and *glpk2* (SCO0509), which encodes a glycerol kinase outside the *gyl* operon. Control of morphological differentiation by AtrA is explained at least in part by transcriptional control of *ssgR* (Fig. 4),[206] the transcriptional activator of the gene encoding SsgA, which is involved in cell division and sporulation.[207,208] Disruption of *atrA* suggests it activates transcription of *ssgR*,[206] and direct binding of AtrA within the upstream regulatory region of *ssgR* was confirmed by ChIP-seq (McDowall *et al.*, unpubl. data).

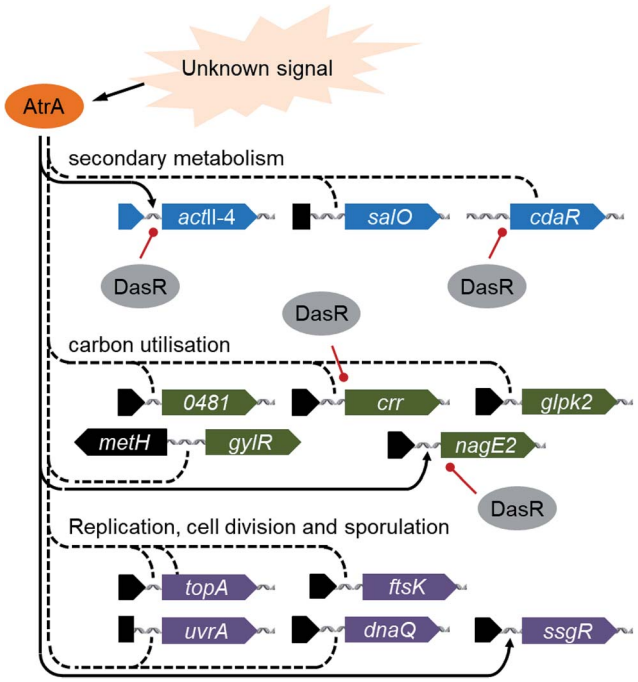

Fig. 4 Schematic illustration of a selection of genes corresponding to sites of AtrA binding in *S. coelicolor*. Black and red solid black lines with arrow heads represent previously described interactions associated with activation by AtrA and repression by DasR, respectively. The broken lines represent interactions identified by chromatin immunoprecipitation but not yet characterized AtrA binds to upstream regions of genes encoding CSRs (*act*II-ORF4, *cdaR* of *S. coelicolor* and *salO* of *S. albus*; the latter encodes the CSR for salinomycin biosynthesis). The activator AtrA and the repressor DasR compete for binding to the upstream regions of *act*II-ORF4 and *cdaR* and upstream regions of genes that are involved in the uptake of GlcNAc (*crr* and *nagE2*). In addition AtrA binds to an upstream region of SCO0481, which encodes a protein that binds chitin, a rich source of GlcNac. The positive control of AtrA on GlcNac uptake suggest that AtrA increases Act production indirectly through enhanced GlcNAc-induced inactivation of DasR as well as directly through activation of *act*II-ORF4 transcription. AtrA also binds to upstream regions of genes involved in glycerol catabolism (*gylR* and *glpk2* (SCO1658)). The binding of AtrA to the upstream region of genes involved in DNA replication (*topA*, DNA topoisomerase 1, *uvrA*, *dnaQ*) cell division and sporulation (*ssgR* and *ftsK*) explains the role of AtrA in the control of morphological development.

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

The signals that are required for activation of AtrA and Rok7B7 are unknown. Since AtrA is a TetR-regulator it is suggested that this protein is regulated in an allosteric manner by a ligand to exert its effect on secondary metabolism. In *S. globisporus*, AtrA is inhibited by the binding of heptaene, a biosynthetic intermediate of lidamycin whose biosynthesis is controlled by AtrA *via* activation of its CSR.[211] As part of this work, it was also reported that the DNA-binding activity of *S. coelicolor* AtrA is regulated by Act.[211] Whilst this finding was shown with different preparations of Act, the specificity of this effect needs to be evaluated further. To our knowledge, in all streptomycetes *atrA* is co-located with a divergent AtrA-target gene (SCO4119 in *S. coelicolor*) that encodes NADH dehydrogenase.[212] There is interest in identifying the substrate of SCO4119 as at least some members of the TetR family interact with ligands that are structurally identical or related to the substrates of proteins encoded by genes divergent to their own.[213] As ChIP-chip experiments failed to show binding of ROK7B7 to genomic DNA under standard growth conditions on minimal media, it was proposed that the regulator requires a co-factor or ligand to facilitate its DNA binding activity. The control of – and gene synteny with – the xylose transport operon *xylEFG* by Rok7B7 hints at C5-sugars as candidate ligands for this regulator.[209]

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

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

6. Connections between phosphate, nitrogen and carbon metabolism

Carbon, nitrogen and phosphate are essential components for the basic building blocks of all cellular life. It is reasonable to assume that acquisition and utilization of these elements would be coordinately controlled. Although widely accepted, molecular characterization of this interconnectivity has only emerged recently, with the important discovery that GlnR, DasR and CRP

jointly regulate three genes for citrate synthesis in the erythromycin producer *S. erythraea*.[216] CRP controls early processes during growth in *Streptomyces* species[106,217] and acts as a global regulator of Act, Cda and Red production, perhaps by coordinating precursor flux.[103] Indeed, 8 out of 22 secondary metabolic clusters on within the *S. coelicolor* genome harbour Crp binding sites, suggesting a pleiotropic role in control of antibiotic production. Further evidence for the connection between C- and N-metabolism *via* GlnR came from elegant experiments showing that several ABC transporter systems are under direct control of GlnR in *S. erythraea*, affecting growth on maltose, mannitol, mannose, sorbitol and trehalose.[218] Recent data show that in *S. coelicolor*, GlnR is activated by glucose,[215] while GlnR directly activates transcription of a putative carbohydrate transport operon *agl3EFG*.[219] Taken together, these data suggest direct linkage between carbon and nitrogen metabolism, albeit perhaps only when certain carbon sources are available.

The understanding of links between nitrogen and phosphate metabolism in *S. coelicolor* is better developed. PhoP and GlnR control antibiotic production in response to the availability of phosphate and nitrogen sources, respectively.[135,220] Similar to the competitive activation of *afsS* by AfsR and PhoP described in Section 3, these two regulators bind to overlapping regions within the *glnR* promoter, but unlike the *afsS* story, PhoP represses *glnR* transcription while only AfsR promotes it[139] (Fig. 1). When phosphate is plentiful, PhoP is inactive and thus AfsR (dependent on the growth phase) activates transcription of *glnR*, but when phosphate is in short supply, PhoP is phosphorylated by PhoR and represses the expression of *glnR* (Fig. 1).[220] In addition, PhoP also directly represses transcription of genes within the GlnR regulon, namely two glutamine synthetases (*glnA* and *glnII*) and the promoter for the *amtB-glnK-glnD* operon, which encodes an ammonium transporter and putative nitrogen sensing/regulatory proteins.[221] Uptake/utilization of nitrogen is presumably superfluous if insufficient phosphate is available, hence the PhoP-mediated repression of genes involved in these processes. Thus, PhoP-mediated control of nitrogen metabolism may help balancing the cellular P/N equilibrium.

Connection between phosphate and carbon metabolism is less well studied, but one link may be governed *via* the PhoP-controlled enzyme PPK (polyphosphate kinase), which affects antibiotic production in response to the level of inorganic phosphate (Pi).[127,222] PPK is involved in maintaining the cellular energy balance by regenerating ATP from ADP and polyphosphates and *ppk* mutants show enhanced Act production under Pi-limited growth conditions.[127] This was recently explained by increased degradation of triacylglycerols (TAGs), resulting in accumulation of the polyketide precursor acetyl-CoA.[223] Additionally, phospho-sugars inhibit antibiotic production in streptomycetes. This effect is mediated by the phosphate- rather than of the glyco-moiety, as the inactivation of *phoP* or *ppk* prevents or enhances, respectively, their utilization as nutrient sources and their inhibitory effect on antibiotic production.[224]

Thus, it is becoming evident that the conventional understanding of the PhoRP, AfsR and GlnR as the elements of the linear transduction systems regulating primary and secondary metabolism have been revised significantly over the last several years. Recent discoveries made it possible to understand, at least partially, the cross-talk occurring between regulators for phosphate and nitrogen metabolism, and to a lesser extent carbon metabolism in streptomycetes. It is a reasonable expectation to predict that established methods for assessing DNA binding *in vivo* (*i.e.* ChIP-seq)[225] in combination with new strategies for robustly mutagenizing and identifying mutants (*i.e.* Tn-Seq)[226] will enhance the ability to probe these regulons and their cross regulation.

7. The impact of metals on secondary metabolism

Iron is an essential metal that plays important roles in DNA replication, protein synthesis and respiration. Iron is relatively unavailable in the soil due to the low solubility of the Fe^{3+} ion under aerobic conditions at neutral pH. Production of iron-chelating compounds called siderophores is the most common way that bacteria circumvent this problem.[227] Moreover, some bacteria have developed systems that allow them to utilize siderophores synthesised by neighbouring microorganisms.[171,228,229] The primary impact of iron deficiency in *Streptomyces* and other bacteria, is the stimulation of siderophore production. All *Streptomyces* species examined thus far appear to harbour a BGC for desferrioxamine, which has been proposed to be part of the 'core' secondary metabolome of the genus,[230] while other streptomycetes produce additional siderophores; *S. coelicolor* and *S. scabies* produce coelichelin and pyochelin, respectively, for example.[231,232] Production of desferrioxamine is normally repressed by the DmdR1 protein, which becomes derepressed in the absence of iron.[233–235] The *dmdR1* gene is unusual in that its DNA sequence encodes a second gene (*adm*) using the anti-sense strand of DNA.[236] Deletion of the *dmdR1-amd* locus in *S. coelicolor* abolished sporulation and the production of Act and Red.[233] Subsequent experimentation whereby either *dmdR1* or *amd* were individually mutated by a point mutation revealed that inactivation of *dmdR1* had no impact on Act and Red production where as these compounds were overproduced when only *amd* was mutated.[236] Another link between iron availability and secondary metabolism in *S. coelicolor* is that iron de-represses the pleiotropic TCS, AbrA1/A2, which negatively regulates Act and Red production, although the mechanism has not yet been resolved.[237]

Zinc is an important transition metal required as a cofactor for many enzymes and regulatory proteins important for normal bacteria physiology. However, the intracellular free level of this element should be maintained within a narrow range due to its potential toxicity.[238,239] Its uptake in streptomycetes as well as in other bacteria is regulated by Zur, a zinc-responsive transcriptional regulator.[240,241] Interestingly, there is a Zur-binding site within the BGC for the metal chelator, coelibactin and adjacent to this is a binding site for another zinc-sensitive regulator, AbsC; together these regulators repress coelibactin biosynthesis.[242] Interestingly, AbsC also seems to be required for the

production of Act and Red when *S. coelicolor* is cultivated under the specific conditions of zinc limitation and inactivation of *zur* and *absC* genes block sporulation. Binding of AtrA upstream of the promoter for *zur*[243] has been identified both biochemically and by ChIP-seq (McDowall *et al.*, unpubl. data) suggesting yet another layer of regulation that potential facilitates integration with primary metabolism as well as secondary metabolism and morphological development. More detailed study of these regulators is necessary in order to fully illuminate their regulons and the nature in which they overlap and interconnect with other metal acquisition systems. *Amycolatopsis japonicum* produces the biodegradable ethylenediame-tetra acetate (EDTA) isomer [*S*,*S*]-EDDS, whose gene cluster was elucidated.[244] Trace amounts of zinc in the culture media inhibit the production of [*S*,*S*]-EDDS, which led to the proposal that the molecule is required for zinc uptake. The synthesis of the zincophore is repressed by the zinc regulator Zur.[244]

Recently, the impact of rare earth elements (REEs) on secondary metabolism was explored. Supplementation of culture medium with scandium or lanthanum stimulated the production Act by *S. coelicolor*, Str by *S. griseus* and actinomycin by *S. antibioticus*.[245] Although precise mechanistic detail is lacking, scandium stimulation of Act production is dependent on the ppGpp synthetase, RelA and is mediated by upregulation of *act*II-ORF4.[245] Interestingly, scandium was also able to rescue the ability of *S. lividans* to produce Act, a compound that the species does not normally produce despite harbouring a nearly identical gene cluster.[245] Quantitative RT-PCR and HPLC analyses showed that in addition to Act, scandium supplementation stimulated the expression of eight other BGCs in *S. coelicolor*.[246] Stimulation of secondary metabolism by REEs is not restricted to Actinobacteria – scandium was recently shown to elicit the production of amylase and bacilysin in *B. subtilis*.[247] Thus, REEs represent a relatively unexplored method for activating the expression of silent or weakly expressed BGCs and future studies should be aimed at understanding the molecular mechanism(s) by which this occurs.

8. Morphological developmental control of antibiotic production

As mentioned in the introduction to this review, the production of antibiotics (and other secondary metabolites) is temporally correlated to the onset of development of *Streptomyces* colonies.[31,33] A model of the linkage between the control of antibiotic production and development is presented in Fig. 5. A likely explanation is that the colony is particularly vulnerable to competitors when it is undergoing programmed cell death (PCD), and antibiotics are produced to protect the colony and the nutrients released during PCD. Until recently, the occurrence of PCD in bacteria has been a subject to major debate, but it is becoming

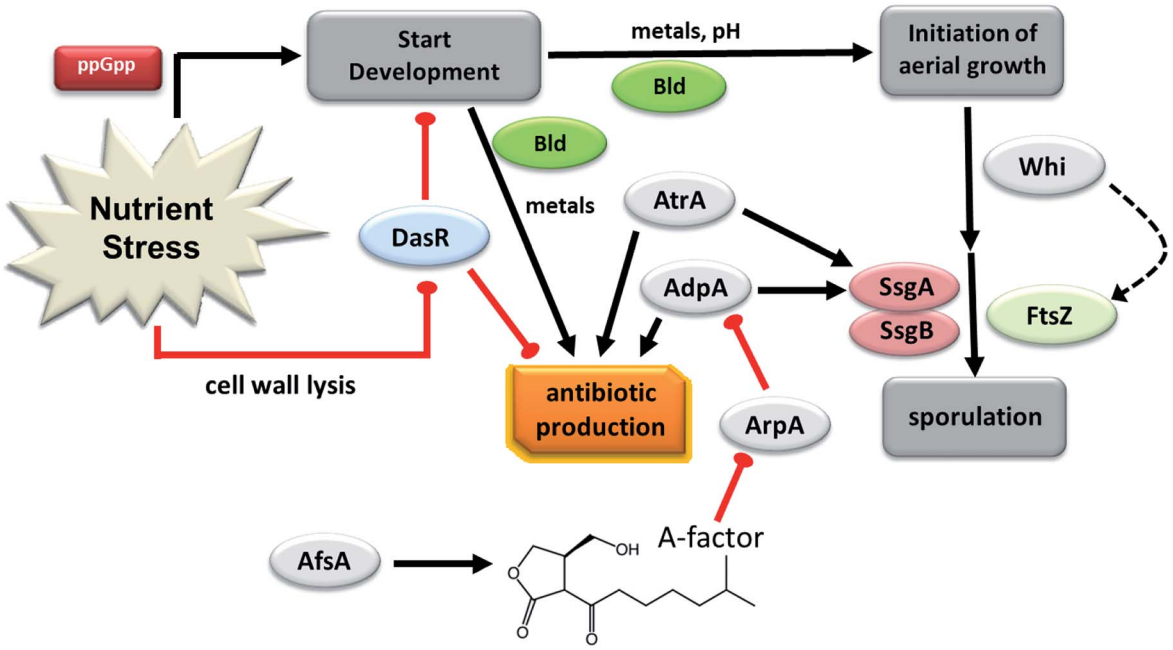

Fig. 5 Initiation of development and antibiotic production. The developmental programme starts with nutrient stress and growth cessation, followed by the accumulation of ppGpp. The autolytic dismantling of the cell wall (PCD) releases cell wall-derived metabolites that inhibit the activity of the nutrient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

increasingly clear that PCD plays a major role the life cycle of multicellular bacteria,[22,248–250] and in that of streptomycetes in particular.[251,252] A direct link between PCD and antibiotic production was demonstrated with the discovery that GlcNAc, which together with *N*-acetylmuramic acid forms the peptidoglycan strands, acts as an elicitor of antibiotic production *via* metabolic inactivation of the global antibiotic repressor DasR.[194,253] For details we refer to Section 5. Interestingly, production of prodiginines, which have anticancer activity by degrading the DNA, may play a direct role in triggering PCD in *S. coelicolor*, and mutants that fail to produce prodiginines have strongly reduced PCD, whereby vegetative growth is prolongued.[254]

As a consequence of the growth phase-dependent control of antibiotic production, developmental mutants that are blocked in an early phase of the life cycle – in particular *bld* mutants – typically fail to produce antibiotics. As mentioned in Section 5.1, mutants of the developmental gene *bldB* are not only disturbed in development and antibiotic production, but are also defective in CCR.[187,188] This links the pathways that regulate carbon utilization and morphological differentiation. BldB is a member of a family of DNA-binding proteins that are only found in Actinobacteria. The family is widespread in streptomycetes, with several paralogues in *S. coelicolor*, including AbaA and WhiJ, which play a role in the control of antibiotic synthesis and development, respectively.[189] Identification of the BldB regulon and the way its activity is modulated will likely offer important new insights into the growth phase-dependent control of antibiotic production and the role of CCR in this process.

BldD is a small DNA-binding protein that is required for development and antibiotic production (Fig. 5).[255] BldD is related to SinR, a master regulator of the transition from the motile to a sessile state in *Bacillus subtilis*, and hence associated with the control of biofilm formation.[256,257] The BldD regulon encompasses over 150 transcriptional units, many of which are involved in the control of development.[258] One of its targets is *bldA*, which at least in part explains the requirement of BldD for antibiotic production. BldD binds to DNA as a homodimer, and dimerization is dependent on the binding of a tetramer of the signalling molecule cyclic-di-GMP.[259] This is another interesting example of small molecule-based control of antibiotic production in *Streptomyces*.

Other *bld* mutants also fail to produce antibiotics, but the phenotype of these mutants is not independent of the growth medium (Fig. 5). In fact, *bldA*, *bldC*, *bldG*, *bldH* (*adpA*), *bldJ* and *bldK* mutants produce spores on non-repressing carbon sources such as mannitol or glycerol, but not on media containing glucose. Interestingly, mutation of *glkA* restores antibiotic production and morphological development to *bldA* mutants,[33] while *bldJ* and *bldK* mutants are rescued by supplementing the colonies with iron. The latter is due to their failure to produce the siderophore desferrioxamine.[170] In fact, most *bld* mutants are affected in desferrioxamine biosynthesis, with strongly reduced production of the siderophore in *bldA*, *bldJ*, and *ptsH* mutants, and overproduction in *bldF*, *bldK*, *crr* and *ptsI* mutants.[170]

An infamous example of translational control of development and antibiotic production is BldA, the tRNA that recognizes the rare UUA codon for leucine. Mutants of *S. coelicolor* defective in *bldA* have a bald phenotype and fail to produce antibiotics.[260,261] The latter is a direct consequence of the presence of UUA codons in the mRNA of the genes for ActII-ORF4 and RedZ.[73,74] The presence of TTA codons in BGCs for specialized metabolites – and in particular in genes encoding CSRs – is more a rule than an exception, which provides strong phylogenetic evidence for the fact that control of antibiotic production by BldA has evolved with a purpose.[262]

Mutants that are blocked in sporulation (so-called *whi* mutants) generally are not affected in antibiotic production. This is most likely because the decisions to switch on secondary metabolism made at an earlier stage in the life cycle. The exception is *ssgA*, whose transcription does not depend on any of the 'classical' *whi* genes.[207] SsgA activates sporulation-specific cell division by controlling the localization of its paralogue SsgB, which in turn recruits FtsZ to initiate sporulation-specific cell division (Fig. 5).[263] In contrast to most developmental control proteins, SsgA and SsgB lack DNA-binding domains. The SsgA-like proteins are unique to sporulating Actinobacteria, and most likely function as chaperones that recruit multicomponent complexes.[264,265] Over-expression of *ssgA* results in overproduction of prodiginines (Red), while Act production is blocked.[266,267] The most likely explanation is that SsgA blocks *S. coelicolor* development at a stage corresponding to early aerial growth, where Red production has been switched on, while Act production has not yet been initiated. SsgA and SsgB probably represent another important link in the coordination of secondary metabolite production with vegetative growth.[268]

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

9. Autoregulators and the control of antibiotic production

Bacteria communicate with each other through production of small extracellular molecules, called bacterial hormones or autoregulators. After the discovery of the gamma-butyrolactone A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), produced by *S. griseus*, many more bacterial hormones have been identified, such as GBLs similar to A-factor, furans, gamma-butenolides and PI-factor. In general, these signalling molecules are active in nanomolar concentrations and diffuse readily

from one actinomycete to another, thereby affecting development and antibiotic production. GBL production is most likely not species-specific, as different species can produce the same GBL, suggesting extensive interspecies communication and 'eavesdropping'. Antibiotics may also function as signalling molecules, thereby induce antibiotic activity and/or resistance, and again in a more general fashion, affecting a broad range of hosts. Thus, the usage of bacterial hormones or antibiotics is an important factor in the discovery of novel antibiotics, as well as co-culturing micro-organisms (recently reviewed in ref. 277).

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

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

ScbR2 is highly similar to ScbR, but unlike ScbR it is not able to bind GBLs, and is hence considered a pseudo gamma-butyrolactone receptor.[278,282] Instead it binds the endogenous antibiotics Act and Red and the exogenous antibiotic jadomycin B and related angucyclines.[278,283] Interestingly, addition of non-endogenous jadomycin B from *S. venezuelae* releases ScbR2 from the promoters of *redD* and *adpA* in *S. coelicolor*, leading to accelerated Red production and morphological differentiation. ScbR2 probably has a greater effect on secondary metabolism than ScbR. Deletion of *scbR2* abolishes Act, Red and Cda production and induces coelimycin production.[281,283] Like ScbR, ScbR2 directly represses *cpkO*.[278] ScbR2 is also a repressor of *scbA*, and acts both directly and indirectly on antibiotic production.[282] ChIP-seq showed that ScbR and ScbR2 have many shared targets genes related to primary and secondary metabolism.[143,284] Both directly act on *afsK* and on genes involved in malonyl-CoA synthesis and hence precursor supply for polyketide natural products. Interestingly, the TetR-like proteins ScbR and ScbR2 can also bind as heterodimers, and co-immunoprecipitation of ScbR2 and ScbR revealed that only the ScbR-ScbR2 heterodimer can control SCO5158, which encodes an uncharacterized protein.[285] Such heterodimer formation is not unique, and was previously proposed for the gene products of *mmfR* and *mmyR* of the methylenomycin BGC.[286]

S. avermitilis contains three GBL-like receptors encoded by genes that are located in a single locus, namely *aveR1*, *aveR2* and *aveR3*. This locus also contains the genes *aco* and *cyp17* required for avenolide biosynthesis. The bacterial hormone avenolide increases avermectin production in a dose-dependent manner when added in nanomolar concentrations to an *aco* deletion mutant.[287] The AveR1 protein was identified as its cognate receptor.[288] Deletion of *aveR1* or addition of avenolide did not influence avermectin production, but increased avenolide production. An explanation for the latter might be that the threshold that is required for avermectin production has already been reached at the start of growth. This led to the suggestion that AveR1 acts as a repressor in the early stages of growth.[289] AverR1 represses its own transcription and that of *aco*.[289]

AveR2 is a pseudo GBL-receptor that represses the transcription of *aveR*, encoding the positive CSR of the *ave* cluster.[290] Additionally, AveR2 represses *aco* and *cyp17*, and controls genes involved in primary metabolism, ribosomal protein synthesis and stress responses. Such an extended regulon is reminiscent of ScbR2 (see above), and it is important to note that both regulators can bind endogenous and exogenous antibiotics. Indeed, the affinity of AveR2 for DNA is influenced by avermectins and also by the exogenous antibiotics jadomycin B and by aminoglycosides. Thus, we note that such pseudo-GBL receptors should be considered as important pleiotropic regulators.[290]

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

9.2. GBL-receptors and antibiotic production in other streptomycetes

The examples of *S. coelicolor* and *S. avermitilis* suggest that the presence of genes for GBLs and their receptor proteins may serve as beacons for cryptic BGCs. Similarly, the BGCs for the angucyclines jadomycin B (from *S. venezuelae*) and auricin (from *S. aureofaciens*) also contain genes for GBL synthases and their cognate receptors.[293,294] The gene *jadR3* harboured within the jadomycin B BGC encodes a putative GBL receptor located upstream of the GBL synthase genes *jadW123*. The product of this GBL synthase system is SVB1, which is identical to the GBL SCB3, produced by *S. coelicolor*. In *S. venezuelae*, only JadW2 is required for jadomycin production.[294] Nevertheless, deletion of *jadW1* abolishes both jadomycin B and chloramphenicol production under conditions that are known to be favourable for production of these antibiotics.[295] JadR3 is an autorepressor and also represses *jadW1* transcription, and thereby represses jadomycin B production.[294] The auricin BGC of *S. aureofaciens* is controlled by the GBL synthase SagA and its cognate receptor

SagR, and again the genes encoding these proteins are located directly next to the biosynthetic genes. Deletion of *sagR* results in early but reduced auricin production, while deletion of *sagA* abolishes auricin production, establishing their key role in controlling auricin biosynthesis. In contrast to other GBL receptor proteins, SagR does not auto-regulate its own transcription, but instead *sagR* and *sagA* are repressed by the CSR Aur1R.[293]

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

The complex regulatory network of the "pristinamycin supercluster" of *S. pristinaespiralis* is also under the control of a GBL-receptor. Pristinamycin is a mixture of two compounds, including the cyclohexane depsipeptide pristinamycin I (PI) and the poly-unsaturated macrolactone pristinamycin II (PII) that are produced in a 30 : 70 ratio. The mixture of pristinamycin is significantly more active against pathogenic bacteria than PI and PII separately.[302] PI is synthesized by non-ribosomal peptide synthetases (NRPS) and PII by hybrid polyketide synthases (PKS)/NRPS.[303] The genes required for PI and PII production are not arranged in a single BGC, but are heterogeneously divided over a 210 kb genomic region whereby the biosynthetic genes are interspersed by a cryptic BGC.[303] These characteristics of the BGC and the fact that the cluster contains seven genes encoding CSRs makes the regulation of pristinamycin biosynthesis very complex.[304] These CSRs include the GBL-receptor SpbR, two TetR-like regulators (PapR3 and PapR5), three SARP regulators (PapRI, PapR2, PapR4) and a response regulator (PapR6).[303,304] The regulatory cascade starts with the release of SpbR from the DNA when its ligand reaches a critical concentration.[304] The pristinamycin BGC is under the direct control of the SARP regulators PapR1, PapR2 and the response regulator PapR6.[304] PapR2 is most likely the master regulator of the pristinamycin BGC, as this is the only regulator that is fully required for pristinamycin biosynthesis.[304] The regulatory genes that directly control the pristinamycin BGC are repressed by the TetR-regulator PapR5.[304,305] PapR5 shows similarity to pseudo-GBL receptors, suggesting that perhaps pristinamycin and/or biosynthetic intermediates act as ligands for PapR5 and may thereby control the level of pristinamycin.[304] Similar as to other regulatory networks, the GBL-receptor is not the first regulator in the regulatory cascade, since SpbR is positively regulated by an AtrA (SSDG_00466) regulator outside the BGC. AtrA in turn positively controls the transcription of PapR5.[305] Thus, the pristinamycin BGC is subject to complex and multi-level control, several elements of which deserve further investigation, so as to unravel the full regulatory network.

10. EMERGING themes in the control of antibiotic production in Actinobacteria

Besides the usual suspects, less well-studied genera of Actinobacteria (often referred to as rare Actinobacteria) also produce a wide range of natural products, and insights into their molecular regulation is important from the perspective of drug discovery and production improvement. Culture collections housed by biotechnology companies and research institutes possess several rare Actinobacteria, including *Micromonosporaceae*, *Streptosporangiae*, *Pseudonocardiaceae*, *Nocardiaceae*, and *Thermomonosporaceae*, and many other rare and unclassified species that have yet to be explored.[306–309] In recent years, interest in strains isolated from marine environments and other ecological niches such as plants and insects has grown because they offer a rich new microbial source for NP discovery.[35,310,311] The regulation of natural product biosynthesis by rare Actinobacteria is poorly characterised, because many of them are genetically intractable and limited genetic tools are available. As the cell wall structure between Actinobacteria often varies and is different from that of streptomycetes, preparation of protoplasts (and regeneration) typically requires different methods.[312] A protocol to prepare protoplasts of *Planobispora rosea*, the producer of the thiazolyl peptide antibiotic GE2270 that targets elongation factor EF-Tu[313] was applied to different rare Actinobacteria.[312] This protocol demonstrated the applicability of both lysozyme and mutanolysin (from *S. globisporus*) to produce protoplasts from these industrially important strains.[312] Other issues that need to be solved for genetic manipulation of rare Actinobacteria include identification of suitable origins of replication for plasmids,[314] the methylation pattern of the DNA[315,316] and the use of specific promoters for expression.[317,318] Many of these technical difficulties can in principle be circumvented by the use of expression of a BGC in a heterologous host. Expression of the BGC for GE2270 of *P. rosea* in *S. coelicolor* M1146 allowed the study of its regulation.[319] Deletion of *pbtR*, encoding a TetR-family regulator, abolished the production of GE2270. Similarly, the BGC for taromycin A from *Saccharomonospora* sp. CNQ490 was also expressed in *S. coelicolor* M1146 to allow its genetic manipulation. Deletion of *tar20*, encoding a LuxR regulator of the taromycin BGC, increased the

production of the compound in the heterologous strain.[320] Heterologous expression of a BGC may often be suitable to study the function of CSRs within a BGC, but for understanding of the global regulatory network and the ecological responses that control the BGC of interest, it is necessary to study the BGC in its natural host. In a number of Actinobacteria, the molecular regulation of antibiotic production has been studied. Especially in strains that produce clinically important antibiotics, such as glycopeptide producers. It appears that the rare Actinobacteria that have been studied indeed contain similar regulators as *Streptomyces* and therefore we expect that most of the control mechanisms of antibiotic production are similar. Below the control of antibiotic production in a number of Actinobacteria is discussed and compared to that of *Streptomyces*.

10.1. Control of glycopeptide biosynthesis

The glycopeptide antibiotics vancomycin and teicoplanin are important last line of defence antibiotics that are used to treat infections associated with multi-drug resistant Gram-positive bacteria.[321,322] Their target is the peptidoglycan precursor lipid II, thereby inhibiting synthesis of the bacterial cell wall.[323] Vancomycin is produced by *Amycolatopsis orientalis* and teicoplanin by *Actinoplanes teichomyceticus*.[324,325] Other well-studied members include the precursor of dalbavancin, A40926 produced by *Nonomuraea* sp. ATCC39727,[326] balhimycin produced by *Amycolatopsis balhimycina*,[327] and the sugarless glycopeptide A47934 produced by *S. toyocaensis*.[328] A comparison of the BGCs for these compounds (*tei* for teicoplanin, *bal* for balhimycin and *dbv* for A40926) and their control is presented in Fig. 6. Members of the glycopeptides share a heptapeptide core, which is synthesized by non-ribosomal peptide synthetases (NRPS), with further modifications such as cross-linking, methylation, halogenation glycosylation or attachment of sulphur groups.[322,329] Glycopeptides bind to the D-alanyl-D-alanine (D-ala-D-ala) terminus of the growing lipid attached peptidoglycan chain on the outside of the cytoplasmic membrane and thereby prevent the binding of transpeptidases that create the cross-links between the polysaccharides, required for cell wall integrity.[323]

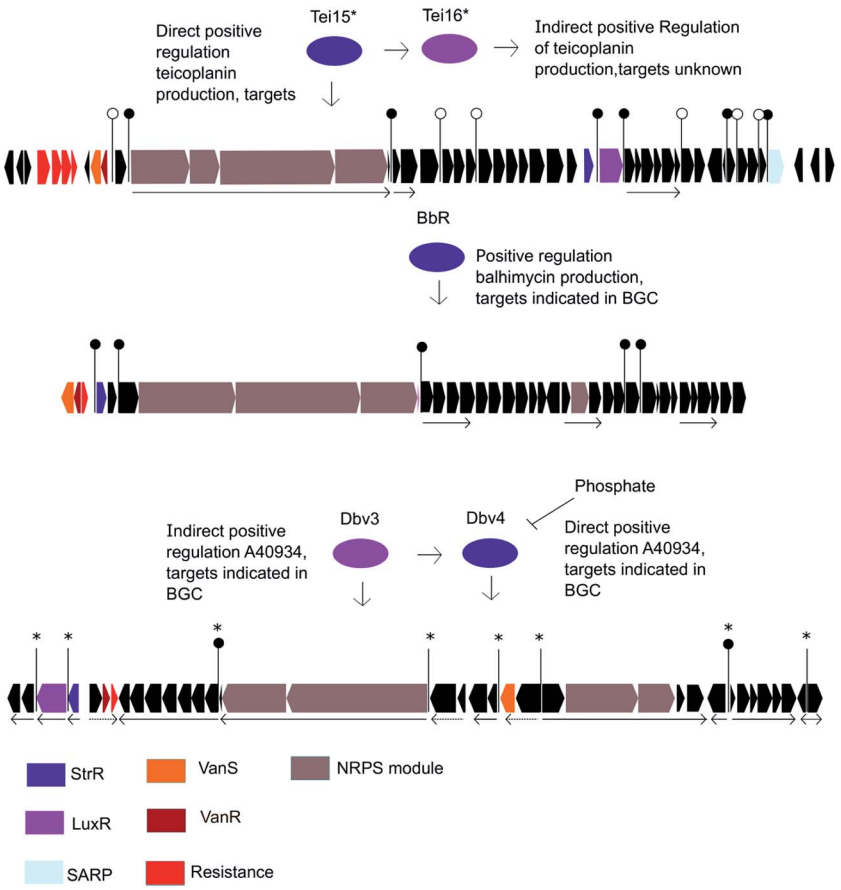

Fig. 6 Regulation of glycopeptide biosynthetic gene clusters. Shown are the BGCs for teicoplanin (*tei*), balhimycin (*bal*) and A40926 (*dbv*). Known and putative binding sites for StrR (purple) are indicated in the clusters with closed and open circles, respectively. The consensus sequence for the StrR binding sites GTCCAR(N)17TTGGAC is shared between all three BGCs. Genes regulated by LuxR (magenta) are indicated with an asterisk. Experimentally confirmed operons are indicated with an arrow. The primary CSR of the teicoplanin BGC is Tei15*, which positively regulates the expression of LuxR-family regulator Tei16* and of the SARP-family regulator Tei31*, with both regulators having unknown targets. The *bal* cluster is regulated by the CSR BbR, and lacks a gene for a LuxR regulator. The primary CSR of the *dbv* cluster is the LuxR regulator Dbv3, which positively regulates the expression of StrR regulator Dbv4, most likely indirectly. For details see the text. BGCs adapted from the MiBIG database.[412]

The BGCs of these antibiotics are typically controlled by CSRs of the StrR and LuxR families.[330–332] The teicoplanin BGC spans 89 kb and includes five regulatory genes, *tei2*, *tei3*, *tei15**, *tei16** and *tei31**.[324,325] Tei2 and Tei3 show high homology with the VanR/VanS system of *S. coelicolor*[333,334] and are involved in the control of teicoplanin resistance. The genes *tei15** and *tei16** encode members of the StrR and LuxR family regulators, respectively. Overexpression of Tei15* results in 30–40-fold increase in teicoplanin biosynthesis.[332,335] Tei15* is the primary CSR, and directly controls the transcription of the regulatory genes *teiA* for the NRPS module, *tei2** (which encodes a deacetylase), *tei16**, *tei17** involved in Dpg synthesis and *tei27** (for an unknown protein). Tei15* also controls the expression of the LuxR family regulator Tei16* and the SARP family regulator Tei31*. The targets of Tei16* and Tei31* in the teicoplanin cluster remain unknown, although Tei16* does positively control teicoplanin production.[332] Tei15* does not show autoregulation, in contrast to its orthologue BbR in the balhimycin BGC.[331,332] See Fig. 6.

The dalbavancin BGC of *Nonomuraea* sp. ATCC39727 contains four regulatory genes, namely *dbv3*, *dbv4*, and the TCS *dbv6* and *dbv22* for the control of resistance (Fig. 6). Dbv4 (similar to StrR and Tei15*) is the likely CSR, and is expressed under phosphate-limiting conditions, while Dbv3 is a LuxR-type regulator similar to Tei16*. Both Dbv3 and Dbv4 are required for A40926 production.[330] Dbv3 controls the transcription of *dbv4*, as well as genes for the biosynthesis of 4-hydroxyphenylglycine, the heptapeptide backbone, and for glycosylation and export. However, similar to the situation for Tei16* in the teicoplanin BGC, no common regulatory elements were identified in the promoter regions of the Dbv3-controlled genes, and control could therefore be indirect.[330] Dbv4 is directly involved in the regulation of genes involved in 3,5-dihydroxyphenylglycine, cross-linking, halogenation, glycosylation and acylation.[330] The gene for Dbv4 and its regulon are repressed by phosphate, whereas Dbv3 and its regulon are not. No Pho-boxes were identified upstream of the *dbv4* genes, suggesting the phosphate repression is indirect.[336]

The glycopeptide balhimycin is produced by *Amycolatopsis balhimycina* (formerly *Amycolatopsis mediterranei*). The balhimycin BGC has a simpler control system with three regulatory genes, namely the VanR/VanS TCS for resistance and the StrR-like regulator Bbr (Fig. 6). Bbr binds to a consensus sequence ($GTCCAR(N)_{17}TTGGAC$) that is found within the promoter for its own transcription, the putative ABC transporter gene *tba*, *oxyA* for a P450 monooxygenase, *dvaA* involved in dehydrovancosamine synthesis and the putative sodium proton antiporter gene orf7.[331] In the three glycopeptide BGCs the StrR CSR binds to the consensus sequence that is conserved in the intergenic regions of the glycopeptide BGCs, although the target sequence may vary and deviate from the consensus.[329,331,332,336] Although these three BGCs are organised in a similar manner and contain regulatory genes, the mechanism of regulation differs between them, and therefore making assumptions about the regulatory network based on bioinformatics alone is not sufficient.[330] In *S. griseus*, StrR is positively controlled by the pleiotropic regulator AdpA. However, overexpression of the putative *adpA* gene of *A. balhimycina* did not induce antibiotic production, although heterologous expression of this regulator in *S. coelicolor*, *S. ghanaensis* and several soil Actinobacteria was successful.[337] Vancomycin biosynthesis and its control are well understood, but the role of StrR regulator in the BGC (AORI_1475) has not been elucidated.

Since most glycopeptide BGCs contain a StrR-like positive regulator, over-expression of the corresponding gene is a logical generic strategy to induce the expression of (cryptic) glycopeptide BGCs. A good example is the production of ristomycin A in *Amycolatopsis japonicum*. This strain is known for the production of (*S*,*S*)-ethylenediamine disuccinic acid [(*S*,*S*)-EDDS], the biodegradable isoform of EDTA (Section 7). Under standard laboratory conditions this strain does not produce antibiotics, but over-expression of the StrR orthologue in *A. japonicum* induced the production of ristomycin A, which is used for the diagnosis of von Willebrand disease and Bernard–Soulier syndrome.[338]

10.2. Control of glycopeptide resistance

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

S. coelicolor is resistant against vancomycin and this resistance is conferred by genes that are similar to the ones present in vancomycin resistant enterococci.[333,334] The resistance cluster of *S. coelicolor* is organized in four transcription units, namely *vanRS*, *vanJ*, *vanK* and *vanHAX*. The latter encode the enzymes required for biosynthesis and incorporation of D-lac in the peptide moiety of the PG. All transcription units are regulated by VanRS.[333] Binding of vancomycin by the N-terminal part of VanS leads to its autophosphorylation, and this phosphate is then transferred to the N-terminal receiver domain of VanR, thereby activating its C-terminal DNA binding effector domain. This results in expression of the resistance genes. In the absence of vancomycin VanS acts a phosphatase that dephosphorylates VanR, and hence *vanS* mutants show constitutive expression of vancomycin resistance.[334,341] In contrast, deletion of *vanS* in *S. toyocaensis* results in sensitivity to A47934, and it was suggested that VanR of *S. coelicolor* is phosphorylated by other proteins while that of *S. toyocaensis* is not.[342] Interestingly, the VanRS TCS is an important determinant of the species-specific glycopeptide resistance profile. *S. coelicolor* is resistant against vancomycin and A47934, but sensitive to teicoplanin, while *S. toyocaensis* is only resistant against A47934.[8] Exchanging the VanRS TCSs between the two *Streptomyces* strains is sufficient to switch the resistance profile.[8] Surprisingly, expression of the VanR orthologue of *A. balhimycina* (VnlR) in *S. coelicolo*r even governed resistance to teicoplanin, and led to increased actinorhodin biosynthesis.[343] VnlR controls *vanHAX* in *S. coelicolor*, despite the fact that it does not control *vanHAX* in *A. balhimycina* itself.[343]

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

An important new element of antibiotic control that was discovered in recent years is the control by σ-factors, the subunits of the RNA polymerase responsible for promoter recognition. An important example is that of the control of lantibiotics. Lantibiotics are ribosomally synthesized, post translationally modified peptide antibiotics (RiPPs[344]). The best known lantibiotic is the food-preservative nisin, produced by *Lactococcus lactis* and discovered as early as 1928.[345] Lantibiotics are synthesized as a prepropeptide encoded by a precursor gene generally referred to as *lanA*. This propeptide is post-translationally modified *via* intramolecular lanthionine bridges that are formed between unusual amino acids to yield the mature peptide.[346] Nisin and several other lantibiotics target the pyrophosphate linkage component of the cell-wall precursor lipid II. As this target is different from that of the clinically used antibiotic vancomycin, there is no cross-resistance with glycopeptides, making them interesting new antibiotics for the treatment of methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[347] Screening a library of 120 000 chemical extracts derived from 40 000 Actinobacteria for activity against cell-wall biosynthesis by Vicuron Pharmaceuticals identified five novel lantibiotics, including microbisporicin (also known and NAI-107) and planosporicin, produced by *Microbispora corallina* and *Planobispora alba*, respectively.[348,349] The control of the BGCs for microbisporicin (*mib* in *M. corallina* and *mlb* in *M.* ATCC-PTA-5024) and for planosporicin (*psp*) have been studied in detail.[350–352] The BGCs have a gene for an extracytoplasmic function (ECF) σ-factor/anti-σ-factor complex (MibX/MibW for microbisporicin and PspX/PspW for planosporicin). ECF σ factors mediate responses to extracellular signals and stress or steps in morphological differentiation,[353,354] but their involvement in the control of antibiotic production was only recognized recently. The microbisporicin and planosporicin BGCs also contain a gene for a regulator with a LuxR-like C-terminal domain. Herein, we use microbisporicin biosynthesis as the example for both BGCs, see Fig. 7 for an overview of its control. The BGC is controlled by its own production by a feed-forward mechanism: deletion of *mibA* results in decreased transcription of the other *mib* genes, while growth of *mibA* mutant colonies adjacent to wild-type microbisporicin-producing colonies restored *mib* transcription.[351,352,355] This effect is specific, since microbisporicin cannot induce the production of planosporicin by *Planobispora alba*.[351] The *mib* cluster includes six transcription units, for synthesis, modification, proteolysis, export, immunity and regulation, and all except the *mibA* structural gene contain the ECF σ-factor promoter motif (GACC-N15-GCTAC) that is recognized by MibX[350,352,355] (Fig. 7). The promoter of *mibA* is controlled by MibR; in turn, transcription of *mibR* depends on MibX and is enhanced by the stringent response. Indeed, deletion of *relA* in *M. corallina* abolishes microbisporicin production. Thus, a complex regulatory network ensures the correct timing of microbisporic biosynthesis, which is induced by both nitrogen starvation and the ensuing stringent response, which activates MibR expression and hence the expression of the (non-toxic)

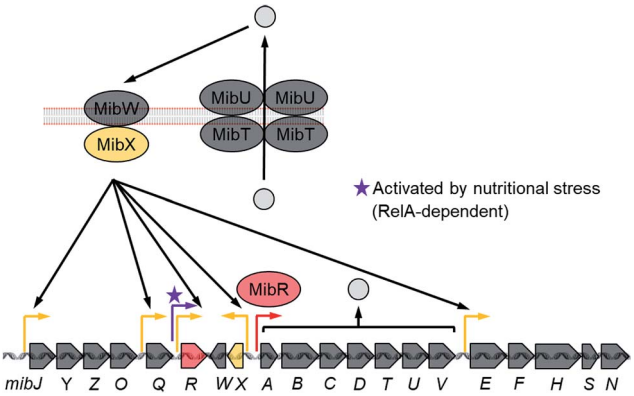

Fig. 7 The regulation of microbisporicin production by *Microbispora corallina*. Nutritional stress leads to the RelA-dependent production of ppGpp which results in the expression of the LuxR-family regulator MibR. MibR activates the expression of *mibABCDTUV*, which results in the production of an immature and less active form of microbisporicin (grey circle) and the means for its export. A basal level of expression of the genes encoding an ECF σ-factor (MibX)/anti-σ-factor (MibW) system enables a feed-forward regulatory mechanism. The immature compound itself or possibly interaction with its lipid II to be sensed by MibW, at which point the ECF σ-factor, MibX is released. MibX then in turn activates its own expression and that of *mibR* as well as the remaining genes in the BGC.

precursor peptide. This precursor is then exported and processed to yield the active antibiotic.[350] Under repressing conditions, MibX is recruited by the membrane bound anti sigma factor MibW, thereby shutting down the biosynthetic pathway. Microbisporicin production also directly depends on the developmental programme, with reduced expression in *bld* mutants, similarly to the biosynthesis of the morphogenic lantibiotic-like morphogen SapB in *S. coelicolor*.[356] For a detailed overview on the regulation of RiPPs in Actinobacteria and other bacterial genera, we refer the reader a recent review.[357]

Involvement of σ factors in the control of antibiotic production is not exclusive to lantibiotic BGCs. SigT regulates Act production in *S. coelicolor via relA* in response to nitrogen starvation, which links nitrogen stress to secondary metabolism.[358] In *S. albus*, the ECF σ^{AntA} controls the synthesis of the antimycin precursor, 3-formamidosalicylate,[359,360] and σ^{25} differentially controls the biosynthesis of oligomycin and of the important anti-helminthic drug avermectin in *S. avermitilis*.[361] Antimycin is a mitochondrial cytochrome c reductase inhibitor produced by diverse Actinobacteria. σ^{AntA} was the first example of regulation of antibiotic production by a cluster-situated ECF σ factor in *Streptomyces* species and it was recently shown that this is likely to be a conserved strategy of regulation for more than 70 antimycin BGCs.[362] Unlike other ECFs, which are controlled by an anti-σ factor that is unable to maintain an inactive complex in the presence of cognate stimulus, σ^{AntA} is an orphan and is not controlled by such a factor. Instead, evidence to date suggests that σ^{AntA} is controlled by Clp proteolysis.[359] The involvement of σ-factor genes in the control of antibiotic production is a new concept, and in particular the presence of σ factor genes within BGCs may function as beacons to identify BGCs in genome mining.

10.4. Regulation of antibiotic production in *Salinispora*

Recently, studies have also been dedicated to the regulatory network of natural product biosynthesis in the marine actinomycete *Salinispora*. *Salinispora* is an obligate marine actinomycete and most of the isolates are derived from marine sediments. The genus knows three different species, under which *S. pacifica*, *S. tropica* and *S. arenicola*.[363] The compounds that were discovered from this genus are predominantly new and therefore this genus is a good example of the concept that new genera derived from remote areas are a good source for the discovery of novel natural products.[363] One of these studies reveals that in *S. tropica* CNB-440, a LuxR-type regulator positively regulates the biosynthesis of the important natural product salinisporamide A, a proteasome inhibitor that is in stage 1 of clinical trials of anti-cancer treatment. This regulator controls the genes involved in the biosynthesis of the salinisporamide A precursor chloroethylmaloyl-CoA, and thereby specifically regulates the production of salinisporamide A and not of other salinosporamides that are produced by *S. tropica* CNB-440.[364]

In the genus *Salinispora* an important concept for the study of cryptic gene clusters was revealed.[365] Transcriptomic comparison of the *Salinispora* strains *S. pacifica* CNT-150, *S. tropica* CNB-440, *S. arenicola* CNS-205 and *S. arenicola* CNS-991 revealed that BGCs common between different strains are not necessarily controlled in the same way and could be active in one while silent in another. Such strain-specific silencing of a BGC was explained by mutation of regulatory genes. Indeed, an orphan BGC in *S. pacifica* (STPKS1) was expressed normally, while its counterpart in *S. tropica* was silent due to the lack of the AraC-family CSR, which was replaced by a transposase. Interestingly, this silent gene cluster is conserved throughout the *S. tropica* clade, which suggests that either this BGC is permanently silenced or that another regulator is involved in the control of the BGC. The BGC for the enediyene PKS1A was silent in CNS-991 and expressed in CNS-205. Comparative genomics and transcriptomic data revealed that a σ factor upstream of the BGC was expressed in *S. arenicola* CNS205, but not in CNS991. Differential expression of this σ factor was proposed be a consequence of its different chromosomal location in the two strains. The BGC for the black spore pigment was present in all four *Salinispora* strains, but the full BGC was only expressed by *S. tropica* CNB-440 and *S. pacifica* CNT-150, whereas only a subset of the genes within the gene cluster was expressed in the two *S. arenicola* strains. The spore pigment BGCs that were entirely expressed contained one or two luxR genes, whereas the partially expressed BGC contained small genes encoding hypothetical proteins of unknown function. The *sta* gene cluster for staurosporine was also differentially expressed between the four *Salinispora* strains, but all strains contained the malT gene for the CSR. Finally, the fact that a BGC (NRPS4) was expressed in *S. arenicola* and *S. pacifica*, but not in *S. tropica* was explained by the lack of a xenobiotic response element in *S. tropica*.[365] Further genetic analysis of these interesting examples is required to fully understand the regulatory mechanisms for these BGCs. The differential expression of gene clusters between different species suggests that one feasible approach to the problem of silent gene clusters may be to look for the same (or highly similar) gene cluster in related Actinobacteria, and see if the cluster is expressed there. With the ever-growing genome sequence information, this approach is becoming increasingly feasible, and is particularly attractive in strains that are not genetically tractable.

10.5. Regulation of rifamycin biosynthesis in *Amycolatopsis mediterranei*

Recently, the molecular regulation of the rifamycin BGC was studied in *Amycolatopsis mediterranei*. Although rifamycin and its derivatives are the first-line anti-tuberculosis drugs, the regulation of the rifamycin BGC was only studied recently. Deletion of *glnR* influences the biosynthesis of rifamycin, although this control is indirect.[366] The LuxR-type regulator RifZ, encoded by the last gene in the gene cluster, positively controls all of the operons in the rifamycin BGC.[367] The rifamycin BGC also encodes a TetR-family repressor (RifQ), which represses rifamycin biosynthesis and efflux. Deletion of *rifQ* resulted in increased production of rifamycin, while accumulation of rifamycin B lowered the affinity of RifQ for its target sequences.[368] This system is consistent with what is known for other TetR-family regulators that control natural product biosynthesis.

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

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

Kitasatospora setae, a member of a genus closely related to *Streptomyces*, harbours several GBL-receptors.[264,372] *K. setae* produces bafilomycins A1 and B1. These macrolides specifically inhibit vacuolar H^+-ATPases and are used in studies of molecular transport in eukaryotes. The genome of *K. setae* contains three genes that are similar to GBL-receptors, namely *ksbA*, *ksbB* and *ksbC*.[373] KsbA binds ^{3}H-labeled SCB1, and deletion of *ksbA* increases bafilomycin biosynthesis.[372] Conversely, KsbC indirectly represses bafilomycin biosynthesis, perhaps *via* the activation of the gene for the autoregulator KsbS4.[373] KsbC also

indirectly activates the production of kitasetaline, a β-carboline alkaloid, and of the kitasetaline derivative JBIR-133.[373]

Interestingly, *Rhodococcus jostii*, a genus of the *Nocardiaceae* produces the GBL (called RJB) that is structurally identical to a precursor of SCB2 (6-dehydro SCB2) produced by *S. coelicolor*, and can bind to the *S. coelicolor* GBL receptor ScbR.[374] This suggests cross-family communication mediated by GBLs in the natural environment. The gene for GBL biosynthesis, gblA, is located in a GBL BGC that is conserved between different *Rhodoccocus* species. This GBL BGC also encodes a GBL-receptor protein GblR and the biosynthesis enzyme GblE, which is an NAD-epimerase/dehydratase. Genome sequencing of *R. jostii* RHA1 indicated that the strain potentially has a rich NP biosynthetic repertoire. The precise role of GBLs in the regulation of natural product biosynthesis in *Rhodococcus*, and the value of the NPs these Actinobacteria can produce, merit further investigation.

11. Outlook

Over the last decade it has become increasingly clear that *Streptomyces* species and other antibiotic-producing Actinobacteria produce only a small percentage of their secondary metabolome under laboratory conditions. Accessing the chemistry specified by this 'silent majority' – also referred to as dark matter – without a doubt holds potential for drug discovery. This untapped resource can be harnessed by both genetic and non-genetic methods which been reviewed recently.[375] The proverbial 'holy grail' in this respect is development of small molecules that can simply be added to culture media to elicit the production of all or ideally only a subset of compounds. Progress has been achieved in this area (*i.e.* sugar-responsive antibiotic repressors, REEs, GBLs and manipulation of C, N and P concentrations, discussed above); the molecular insights that is reviewed above can be harnessed to develop strategies to activate antibiotic production. Clearly, more work is required with the identification of other small molecules. Reporter-based methods have therefore been developed to aid detection of activated or de-repressed gene clusters,[376,377] and screening using small molecule libraries forms an attractive black box alternative to rational approaches that are based on molecular insights.[378,379] For details on molecular, environmental and HT screening approaches to find elicitors we refer the reader to recent reviews.[35,380] Elicitors are also instrumental in unsupervised metabolomics approaches, required to identify compounds in the complex metabolic matrix of microbial cultures.[381] Here, significant fluctuation of the secondary metabolome needs to be achieved, allowing statistical correlation of a given bioactivity of interest to a specific metabolite and/or a BGC. NMR- or MS-based metabolomics then facilitate the identification of the sought-after bioactive molecules.[382–385]

Ultimately, the productivity of any given biosynthetic pathway is dictated by one or more CSRs. The examples provided by among others *Salinispora* show that BGCs may be silent in one species of a given genus, and active in another. Thus, with the growing wealth of genome sequence information, a promising strategy is to look for related bacteria that harbour a close relative of the gene cluster of interest. Indeed, it is not illogical to assume that over the hundreds of millions of years of evolution, the natural products specified by the BGCs have remained structurally the same or highly similar, but are expressed under different growth conditions or in response to different environmental stimuli. The functionality of most putative CSRs can be deduced bioinformatically (*i.e.* as a repressor or an activator). Therefore, an obvious strategy and one that is commonly employed for elicitation of poorly expressed BGCs is augmentation of endogenous regulatory system(s). For example, by deleting genes encoding repressors or over-expressing those encoding activators.[232,386] This strategy depends upon the genetic tractability of the organism, but this is becoming less and less of a requirement as the cloning of large genomic fragments and their *de novo* synthesis becomes more feasible, which enables their tractability and heterologous expression in a panel of potential hosts.[387–389] Indeed, it is now possible to completely refactor the regulation of a biosynthetic pathway by replacing native promoters with those that are constitutively expressed to increase production titres using CRISPR-Cas9 technology.[390] Longer term, improved understanding of how secondary metabolism is controlled and the development of approaches to exploit this and/or efficient synthetic biology strategies to activate biosynthetic pathways are required in order to capitalise on the treasures beneath our feet.

12. Conflicts of interest

The authors have no conflicts to declare.

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