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Review

A brief tour of myxobacterial secondary metabolism

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ABSTRACT

Myxobacteria are soil-dwelling, Gram-negative bacteria which are notable not only for their multi-cellular 'social' lifestyles, but for production of structurally diverse secondary metabolites with potential in clinical therapy. Here we briefly review the history of myxobacterial natural products research, provide an overview of their unique secondary metabolism, with an emphasis on assembly line biosynthesis of polyketide and non-ribosomal peptide metabolites, and look to the future of the field.

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1. Introduction

Natural products have for many years formed the bedrock of modern drug discovery programs: fully 34% of all small-molecule drugs approved worldwide during the period 1981–2006 were natural products or their close semi-synthetic derivatives.[1] Indisputably, the star producers of secondary metabolites are members of the Actinomycetales (ca. 8000 compounds characterized to date), notable among them the *Streptomyces*, followed closely by the genus *Bacillus* (1400) as well as the pseudomonads (400).[2] Over the last decade, however, the myxobacteria have emerged as a promising alternative source for bioactive molecules.[3] Myxobacterial natural products exhibit many unique structural features relative to other metabolites, as well as rare or novel modes of action, making them attractive lead structures for drug development. In addition, the complex biosynthesis of many of these compounds deviates significantly from established precedents in other bacteria. Here we aim to summarize the unusual aspects of myxobacterial secondary metabolism, as well as highlighting the therapeutic promise of these fascinating natural products.

2. Why has myxobacterial secondary metabolism been underappreciated?

In 1892, Ronald Thaxter first recognized the myxobacteria as a distinct group of organisms.[4] Although their peculiar behavioral

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and morphological characteristics have fascinated microbiologists ever since, fundamental knowledge of their basic physiology, biochemistry and genetics has only emerged over the last several decades. Multiple features of myxobacterial microbiology help to explain the sluggish pace of progress in this field, relative to studies of other natural product producers.[2] Because myxobacteria divide very slowly under laboratory conditions (4–14 h doubling time), faster growing microbes such as the actinomycetes were identified first in soil samples. In addition, most strains grow as lumps and flakes when first inoculated, with homogenous suspensions produced only after weeks or months of repeated re-culturing. As full structure elucidation of natural products often requires fermentation-scale growth of the producing organisms, these characteristics significantly impeded and continue to impact attempts to identify the myxobacterial secondary metabolome.

Genetic manipulation of the producing strains is also difficult. Plating on solid medium seldom yields single colonies in high numbers, and when colonies emerge, they grow only slowly (8–14 days). Furthermore, techniques developed for one strain often cannot be applied directly to another, even if the two strains are phylogenetically closely related. In addition, no autonomously-replicating plasmids for myxobacteria have been reported until recently,[5,6] and most strains exhibit natural multi-resistance to commonly-used antibiotics, hampering the identification of suitable resistance markers. Nonetheless, the first biosynthetic gene cluster—that for saframycin Mx1[7]—was identified in 1995, ushering in the molecular age of myxobacterial secondary metabolism.[8] Twenty three complete gene clusters have now been reported from myxobacteria, with new loci discovered every year (6 since 2006).[9–14]

3. Why are myxobacteria multi-producers of secondary metabolites?

To date, only a few microorganisms have been identified as good producers of natural products: yeast and other fungi,[15] as well as groups of bacteria[2] (species of *Bacillus*, the pseudomonads, the actinomycetales, the cyanobacteria,[16,17] the myxobacteria, and the insect pathogenic bacteria[18–20]). It is therefore reasonable to ask why the myxobacteria exhibit a diverse secondary metabolism (although answers to this question remain in the realm of speculation). With the exception of a few marine strains,[21] the majority of myxobacteria have been isolated from the soil, a habitat which is rich in both organic matter and microbial life, including fungi and actinomycetes. Although myxobacterial cells can move by gliding or creeping over surfaces,[22] within the context of the swarm, they remain virtually stationary. Therefore, one reasonable explanation for myxobacterial productivity is that they are simply 'keeping up with the Jones'—to protect its ecological niche in the highly competitive terrestrial environment, each species must maintain an armament of antibacterial and anti-fungal agents. Compound production rates are typically highest during the exponential phase of growth (Ref. 22 and C. Kegler, R.M., unpublished data). This behavior contrasts with that of the actinomycetes, in which secondary metabolism correlates with the onset of the statonary phase.[23] The ability to not only fend off your neighbors but to actively kill them—if concentrations reached in the natural environment are sufficient—might be particularly useful for some myxobacterial strains such as *Myxococcus*, which are capable of degrading proteins and even whole cells of other microorganisms, through excretion of exoenzymes.[24] Alternatively (or in addition), a major role of myxobacterial small molecules may be in modulating cell–cell interactions within the enormously complex soil communities.[25,26] Indeed, recent studies have demonstrated the critical roles played by the metabolites DKxanthene[13] and stigmolone[27] in the developmental cycles of *Myxococcus xanthus* and *Stigmatella aurantiaca*, respectively.

4. Myxobacteria and their secondary metabolism—an overview

Due primarily to the work of the Höfle and Reichenbach research groups at the HZI Braunschweig, Germany (formally the German Research Center for Biotechnology (GBF)), more than 7500 different myxobacterial strains have been isolated to date, all of which are currently housed in the institute's collection and at the German Type Culture Collection (DSMZ). Within the order Myxococcales, 50% of strains belong to the suborder *Cystobacterineae* (with *Cystobacter* the most prevalent genus), 36% to the *Sorangiineae* (68% of which are species of *Polyangium* and *Chondromyces*), and the remaining 14% to the newly-established suborder *Nannocystineae* (here species of *Nannocystis* predominate (43%)).

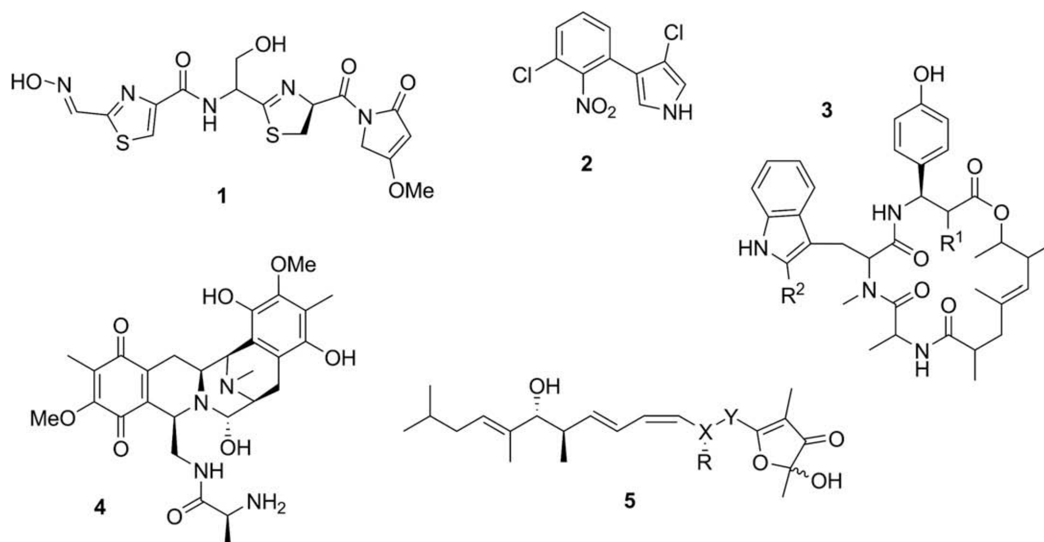

Figure 1. Myxobacterial secondary metabolites. Althiomycin **1** and pyrrolnitrin **2** are known from other bacteria, while the chondramides **3**, saframycin Mx1 **4** and the aurafurones **5**, share close structural similarity to previously identified metabolites from the marine environment and from *Streptomyces*. Chondramide A, R^1 = OMe, R^2 = H; chondramide B, R^1 = OMe, R^2 = Cl; chondramide C, R^1 = H, R^2 = H; chondramide D, R^1 = H, R^2 = Cl. Aurafuron A, R = OH, X–Y = CH_2–CH_2; aurafuron B, R = H, X–Y = CH=CH.

(Ref. 28 and R Garcia, R. M., unpublished observations) Although many more Actinomycetes than myxobacteria have been discovered to date, the known myxobacterial strains have yielded at least 100 natural product core structures, and some 500 derivatives. Secondary metabolism does not appear to be a shared property of all myxobacteria, however, as the majority of compounds have been isolated from select species, including strains of *Sorangium cellulosum*, *Myxococcus xanthus*, and *Chondromyces* species. However, these findings may to some extent reflect productivity under laboratory culturing conditions, and not the true genetic potential of all analyzed strains.

At the time of their discovery, many of the metabolites were novel. Only a few, such as althiomycin **1**[29,30] and pyrrolnitrin **2**[31,32] (Fig. 1), had previously been identified in other bacteria including *Streptomyces* and the pseudomonads. More commonly, the structures were new, but shared architectural elements with previously characterized natural products from both terrestrial and marine sources. For example, the chondramides **3** produced by *Chondromyces crocatus* strains, closely resemble the jaspamides isolated from marine sponges of the genus *Jaspis*[33] (suggesting that the true producer may be a marine myxobacterium), while myxobacterial saframycin Mx1 **4** and the aurafurones **5** (Fig. 1) are almost identical to compounds found in *Streptomyces*.[9,34–36]

4.1. Bioactivity and mode of action

Many myxobacterial compounds exhibit antibacterial (29%) or anti-fungal (54%) properties, likely reflecting the competitive pressures of their natural environments. However, these natural products show a much wider range of biological activities which are often less straightforward to rationalize.[2] Rare but notable properties include anti-malarial, immunosuppressive, insecticidal, and herbicidal activities, while a much more significant number of compounds exhibit cytostatic or cytotoxic effects against eukaryotic cell lines.

The mode of action of multiple metabolites has been investigated, and in many cases, the compounds were shown to target cellular structures which are rarely hit by other metabolites. Twenty unique myxobacterial structures[2] inhibit mitochondrial respiration, including aurachin C **6**,[37,38] the crocacins **7**,[39] and the closely related bithiazole metabolites, cystothiazol,[40] myxothiazol **8**[41,42] and melithiazol **9** (Fig. 2).[43,44] The compounds act at different stages of the respiratory chain, blocking both complex I (NADH:ubiquinone oxidoreductase) and complex III (bc_1-complex). The selective activity of stigmatellin and myxothiazol towards complex III has been exploited in 'chemical genetics' studies to elucidate the biochemistry of the respiratory pathway,[2] culminating in the recent publication of the crystal structure of a bc_1-stigmatellin complex.[45] Stigmatellin also inhibits plant photosynthesis at the b_6/f complex,[46,47] while the aurachins target the corresponding pathway in cyanobacteria.[48]

At nanomolar concentrations, the *Sorangium cellulosum* metabolite soraphen A **10** (Fig. 2) potently inhibits the biotin carboxylase (BC) domain of human, yeast and other eukaryotic acetyl-coenzyme A carboxylases (ACCs), an entirely novel mode of action. As the survival of tumor cells depends heavily on the activity of ACCs, specific inhibitors of this enzyme show promise as therapeutic agents for cancer.[49] The utility of soraphen A, however, is limited by its poor water solubility and low bioavailability, but one of the 49 known variants of this metabolite,[2] or alternatively synthetic or genetically engineered derivatives, might address these formulation issues while retaining bioactivity.

Other myxobacterial compounds bind to DNA (e.g., saframycin),[34] alter the osmoregulation of fungi (e.g., ambruticin),[50] and inhibit eukaryotic (e.g., gephyronic acid)[51] and prokaryotic (e.g., myxovalargin)[52,53] protein synthesis, as well as viral nucleic acid polymerases (e.g., etnangien).[54] Etnangien is also one of a number of metabolites which target eubacterial RNA polymerases.[54] Inhibition occurs both during initiation (sorangicins[55,56]) and chain elongation (ripostatins,[57,58] and the chemically-related myxopyronins[59] and corallopyronins[60]). Although several other inhibitors of RNA polymerase are known (including thiolutin,[61,62] holomycin[63] and streptolydigin[64]), the only class of compounds used clinically is the rifampicins. These molecules also target the initiation of RNA synthesis.[65] Intriguingly, ripostatin and corallopyronin show no significant cross-resistance with rifampicin, and are therefore likely to act by a different mechanism. This observation suggests the potential utility of these metabolites or their derivatives in overcoming rifampicin-resistant bacteria,[66] although to date the compounds have not been developed further.

Small molecules which interfere with microtubule assembly play a critical role in currently available cancer chemotherapies, through the inhibition of cell proliferation and the induction of apoptosis. This fact accounts for the significant interest in the 10% of myxobacterial compounds which target the eukaryotic cytoskeleton. Several myxobacterial natural products work specifically on actin, including rhizopodin **11** (Fig. 2)[67,68] and the chondramides **3** (Fig. 1)[33,69] The chondramides appear to have the same binding site on actin as phalloidin, the notorious toxin of green and white deathcap mushrooms, but effectively penetrate mammalian cells, resulting in IC_{50} values in the low nanomolar range.[70] The natural chondramide variants all have similar activity, although chondramide C is the most effective.[69]

Agents which interact with microtubules can be divided into two classes; the first class of compounds inhibits polymerization of tubulin (at least in vitro), while the second class favors it, thereby interrupting the disassembly process. Among myxobacterial metabolites are compounds which exhibit both modes of action (Fig. 2): the epothilones **12**[71,72] induce tubulin polymerization and stabilize microtubules, while the tubulysins **13**[73,74] accelerate depolymerization. Significantly, the epothilones and their analogues have demonstrated antitumor activity towards multidrug-resistant and paclitaxel-resistant tumor cell lines, both in vitro and in vivo.[75] The semi-synthetic epothilone derivative ixabepilone (Ixempra™) was approved by the FDA in 2007 for the treatment of metastatic breast cancer, while several other compounds including the natural products epothilones B and D, are currently in clinical trials.[76] The tubulysins, of which tubulysin D shows highest potency, exhibit activity which exceeds that of other tubulin modifiers, including the epothilones, vinblastine and taxol, by 20- to 100-fold.[77,78] Ongoing SAR studies of tubulysin D are beginning to define the structural features required for toxicity, as well as suggesting strategies for tailoring the metabolite's pharmacological properties.[79] Meanwhile, research on a second derivative, tubulysin A, has demonstrated its potential both as an antiangiogenic and antiproliferative agent.[80]

4.2. Structural diversity

The representation of metabolite structural classes in myxobacteria is striking. Steroid synthesis is extremely rare in bacteria, but both cholestenols[81] and lanosterol[2] have been isolated from myxobacterial extracts. Myxobacteria are also known to produce two different iron transport metabolites, the hydroxamate-type nannochelins,[82] and the catecholate-type myxochelins A and B,[83–85] ceramides and cerebrosides, as well as carotenoid glycosides esterified with fatty acids.[2] Biosynthesis of the earthy-smelling sesquiterpenoid geosmin **14** (Fig. 3) by certain strains of myxobacteria was described in 1981.[86] More intensive recent analysis of strains of *Stigmatella aurantiaca*[87] and *Myxococcus xanthus*,[88] has revealed a much larger number of volatile substances from different compound classes, including ketones, esters, lactones, sulfur and

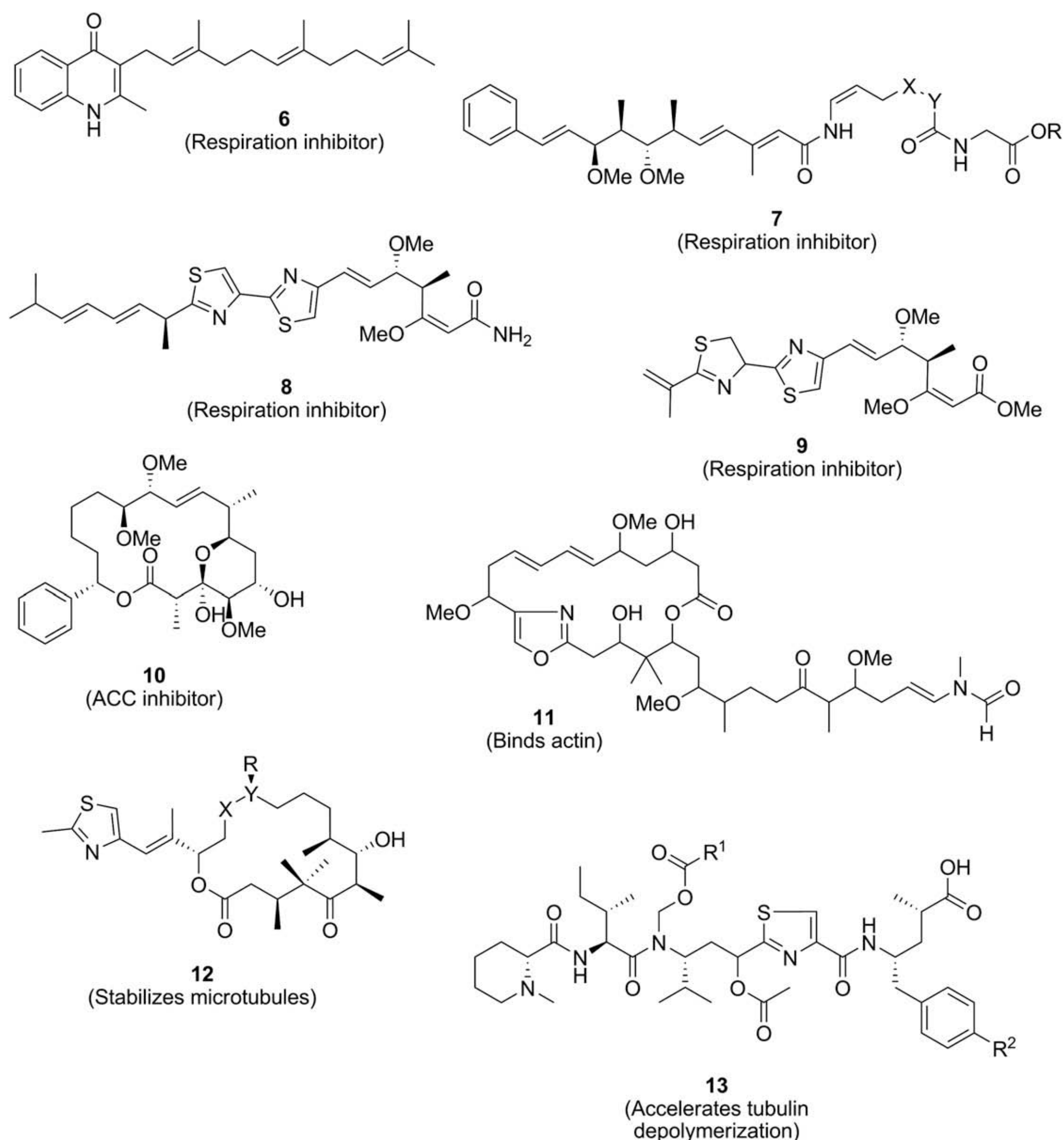

Figure 2. Bioactivities of selected myxobacterial metabolites. Aurachin C **6**, the crocacins **7**, myxothiazol **8**, and melithiazol **9**, inhibit mitochondrial respiration. Crocacin A, X–Y = CH═CH, R = Me; Crocacin B, X–Y = CH═CH, R = H; Crocacin D, X–Y = CH_2–CH_2, R = Me. Soraphen A **10** exhibits a unique mode of action, targeting eukaryotic acetyl-coenzyme A carboxylases (ACCs). 10% of myxobacterial metabolites interact with the cytoskeleton. Rhizopodin **11** binds to actin, while the epothilones **12** and the tubulysins **13**, exhibit opposite effects on tubulysin polymerization. Epothilone A, R = H, X–Y = epoxide; epothilone B, R = Me, X–Y = epoxide; epothilone C, R = H, X–Y = CH═CH; epothilone D, R = Me, X–Y = CH═CH. Tubulysin A, $R^1 = CH_2CH(CH_3)_2$, R^2 = OH; tubulysin B, $R^1 = CH_2CH_2CH_3$, R^2 = OH; tubulysin D, $R^1 = CH_2CH(CH_3)_2$, R^2 = H; tubulysin E, $R^1 = CH_2CH_2CH_3$, R^2 = H.

nitrogen-containing molecules, and additional terpenes, some of which were novel (Fig. 3). However, the vast majority of metabolites discovered to date are linear or cyclic polyketides (PKs) and non-ribosomal polypeptides (NRPs). Intriguingly, more than 50% of the compounds incorporate both PK and NRP elements (and are therefore termed hybrid PK/NRP metabolites), in contrast to the preponderance of purely PK or NRP natural products synthesized by the actinomycetes and *Bacilli*. In fact, only 4 solely NRP metabolites (or metabolite families) have been reported to date (the argyrins **15**,[89] myxovalargins,[53] vioprolides,[90] and thiangazole **16**[91] (Fig. 4)). The evolutionary origin of this biased metabolite profile remains unclear at present.[92]

Closer inspection of myxobacterial PK, NRP and hybrid metabolites reveals many unusual structural features. In actinomycetes, one common method for diversifying the structures is to perform various tailoring reactions (e.g., oxidation, reduction, acylation and glycosylation), after formation of the core molecules;[93,94] these modifications are often crucial for conferring bioactivity on the structures. In contrast, late-stage modification reactions of myxobacterial metabolites, particularly glycosylation, are relatively rare. One exception, however, is the addition of 6-deoxyglucose to the aglycone of chivosazol.[95,96] However, significant structural diversity is present within the basic scaffolds of myxobacterial compounds. A notable example is the leupyrrin family of metabolites **17** (Fig. 5),[97] which was isolated from several strains of *Sorangium cellulosum*. The leupyrrins not only incorporate PK and NRP building blocks, but an isoprenoid unit and a dicarboxylic acid[98]—a natural example of 'combinatorial biosynthesis.'[99] Chemical functionalities of note within other myxobacterial compounds (Fig. 2) include the β-methoxyacrylates of myxothiazol[100,101] and melithiazol;[44,102] the benzoyl-CoA starter unit of soraphen A;[103,104] the nitro moiety of pyrrolnitrin;[31] the cyclopropane ring

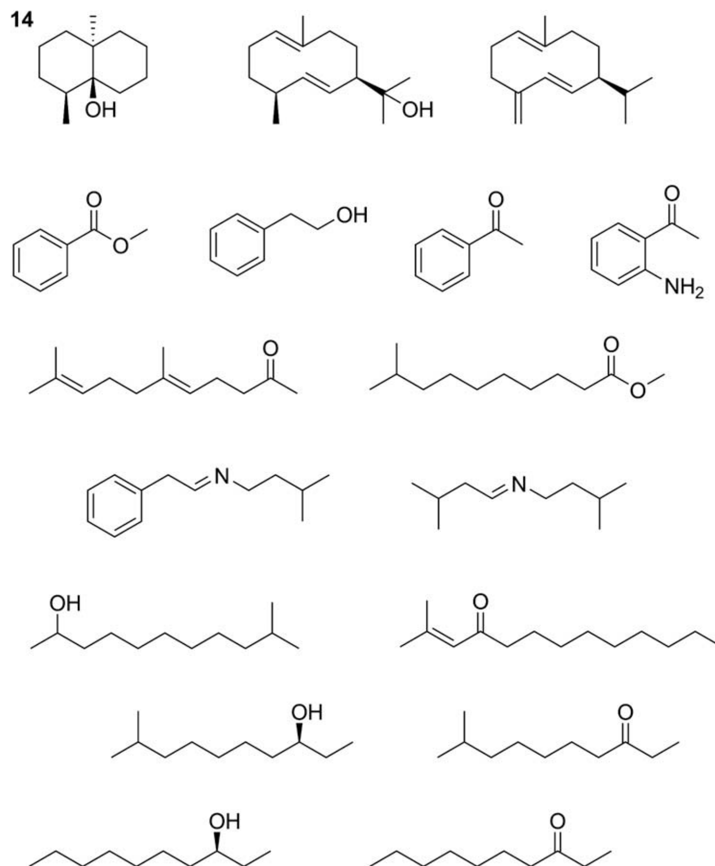

Figure 3. A selection of volatile substances produced by the myxobacteria *Stigmatella aurantiaca* and *Myxococcus xanthus*, including the musty-smelling sesquiterpenoid geosmin **14**.

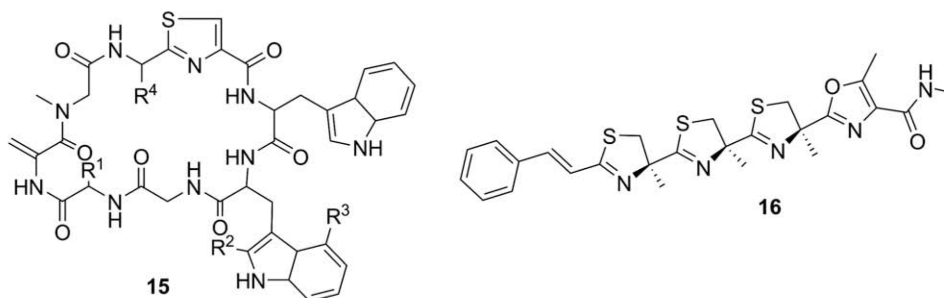

Figure 4. The argyrins **15** and thiangazole **16** are among the few myxobacterial metabolites which are constructed exclusively from amino acid building blocks. Argyrin A, R^1 = Me, R^2 = H, R^3 = OMe, R^4 = Me; Argyrin B, R^1 = CH_2CH_3, R^2 = H, R^3 = OMe, R^4 = Me; Argyrin C, R^1 = Me, R^2 = Me, R^3 = OMe, R^4 = Me; argyrin D, R^1 = CH_2CH_3, R^2 = Me, R^3 = OMe, R^4 = Me; Argyrin E, R^1 = Me, R^2 = H, R^3 = H, R^4 = Me; argyrin F, R^1 = Me, R^2 = H, R^3 = OMe, R^4 = CH_2OH; Argyrin G, R^1 = CH_2CH_3, R^2 = H, R^3 = OMe, R^4 = CH_2OH; Argyrin H, R^1 = Me, R^2 = H, R^3 = OMe, R^4 = H.

of ambruticin;[10,105] the chlorinated tyrosine of chondrochloren;[106] the (*R*)-β-tyrosine[107] and chlorotryptophan residues of the chondramides);[108] the spiroketal of the spirangienes;[11] the aromatic chromone and isochromanone rings of stigmatellin[109,110] and ajudazol,[111] respectively; and the *trans*-(2*S*,4*R*)-4-methylazetidine-carboxylic acid of vioprolide.[90] The myxobacterial metabolite tartrolon B is one of only four natural products known to complex boron.[2,112]

Another characteristic feature of myxobacterial secondary metabolism is the presence in many strains of compound families, which bear little overall resemblance to each other. For example, strains of *Chondromyces crocatus* produce in parallel six or seven distinct classes of metabolites (e.g., the ajudazols, chondramides, chondrochlorens, crocacins, crocapeptins, and thuggacins in strain *C. crocatus* Cm c5[33,39,106,108,111,113]). Family units range in size from small (2 members) to extremely large: a single strain of *Sorangium cellulosum* produces nearly 50 different soraphens.[2] In this case, structural variety arises through the choice of building blocks used during the biosynthesis and the extent of reduction at various positions in the macrolide (with added variation introduced through post-assembly oxidation and methylation). Thus, in the absence of an extensive arsenal of modifying enzymes, myxobacteria manage to diversify the chain building process itself (mechanisms by which this might occur are discussed in Section 5). Interestingly, the composition of metabolite families varies among strains within the same species, so that the ability to synthesize a certain compound is normally a strain-, and not a species-specific property. For example, one strain of *Myxococcus fulvus* makes 30 myxothia-

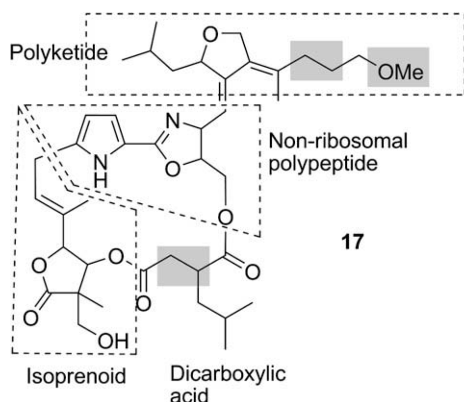

Figure 5. Structures of the leupyrrins **17**. The leupyrrin family of compounds incorporates polyketide and non-ribosomal peptide building blocks, as well as a rearranged isoprenoid unit and a dicarboxylic acid. Sites of structural variation among family members are indicated by grey blocks.

zoles, while another produces only two; one *Sorangium cellulosum* strain synthesizes sorangicin, disorazol, chivosazol and sulfangolid, while another makes disorazol, icumazol and soraphen.[2] A possible explanation for this high intraspecies variation is that secondary metabolites may play a role in cell-to-cell communication, within tightly-knit soil communities.[114] Often, the major component within each family is the most active, suggesting that selection has also favored biological efficacy. Indeed, many semi-synthetic derivatives of myxobacterial metabolites are less potent than their parent compounds.[2]

4.3. Is the myxobacterial secondary metabolome exhausted?

One criticism often leveled at natural products drug discovery is the diminishing returns from screening of microbial extracts: repeated rediscovery of the same compounds renders the process ineffective.[115,116] However, what is also clear is that bacteria rarely fulfill their full metabolic potential under standard laboratory conditions.[117] This appears to be the case with myxobacteria. For example, *Myxococcus xanthus* strain DK1622 is only known to produce five different metabolites: the mixed PK-NRP myxochromides,[118,119] myxalamids,[120] myxovirescins,[121,122] and DKxanthenes,[13] and the siderophore myxochelins.[83,84] Literature reports of additional metabolites from other *Myxococcus xanthus* strains are limited to the NRP metabolites cittilin,[123] saframycin Mx1[34] and althiomycin.[30] However, the recent sequencing of the DK1622 genome has revealed that its metabolic capability is considerably greater, with 8.5% of the genome dedicated to natural product biosynthesis[125] (vs. 4.5% in *Streptomyces coelicolor*[126] and 6.6% in *Streptomyces avermitilis*[127]). The strain contains at least 18 gene clusters encoding PKS, NRPS or hybrid systems. Similarly, three metabolite families have been identified in *Sorangium cellulosum* So ce56 by traditional screening methods, the etnangiens,[54] the chivosazols[95,128] and the myxochelins,[83,84] but its genome contains a total of 17 loci involved in secondary metabolism. As well as additional PKS (including two type III PKS)[129] and NRPS genes, coding regions are also present for carotenoid and terpenoid biosynthesis. Expression of one of the type III PKS in the heterologous host *Pseudomonas putida* resulted in production of flaviolin,[130] although the compound had never been isolated from So ce56 (or any other myxobacterium), demonstrating that the gene is functional. Furthermore, analysis of the *Myxococcus xanthus* DK1622 proteome[131] has shown that most of the clusters of unknown function are expressed in the strain, suggesting that the unassigned genes present in *Sorangium cellulosum* are also active. If these strains are representative of other myxobacteria, it appears that after decades of discovery efforts, we have only begun to scratch the surface of myxobacterial secondary metabolism.

In support of this hypothesis, comparative analysis by UPLC-coupled high resolution ESI-TOF mass spectrometry of the metabolic profiles of 98 *Myxococcus xanthus* strains originating from locations worldwide, revealed 37 candidates for novel compounds (although some may be new derivatives of previously identified metabolites).[114] Even within a single soil locality (16×16 cm), the intraspecific metabolic variation was astonishing. Comparative metabolic profiling of the co-localized strains revealed that many produced a similar complement of compounds, but that the amount of each metabolite present in the strains was significantly different. This strain-specific metabolism argues for wide-ranging efforts to culture and analyze many strains from each species, in order to determine its real metabolic potential. Evidently, much remains to be learned about the true extent of myxobacterial metabolic activity.

5. Peculiarities of megaenzyme biosynthesis in myxobacteria

Myxobacteria excel in producing complex polyketide and non-ribosomal peptide structures. Both classes of metabolites are assembled on gigantic multienzyme proteins, called polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), respectively.[132–134] In keeping with this biosynthetic logic, hybrid PK-NRP metabolites are constructed by hybrid PKS-NRPS systems. In each case, biosynthesis proceeds through the coupling of simple building blocks, acyl-CoA thioesters in the case of the PK metabolites, and both proteinogenic and non-proteinogenic amino acids, in the case of NRPs. The chain extension intermediates undergo various processing reactions during the assembly process, and may be further modified once they are released from the multienzymes.

According to the biosynthetic paradigm developed on the basis of actinomycete and *Bacillus* systems, PKS and NRPS multienzymes function like molecular assembly lines. Each round of chain extension is accomplished by a specific module of enzymatic domains, with each domain catalyzing a particular step (Fig. 6). A PKS module minimally comprises an acyl transferase (AT) for selection of the specific building block, a ketosynthase (KS) to catalyze carbon–carbon bond formation, and an acyl carrier protein (ACP), to which the chain is tethered during its construction. Optional domains include ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) functions, which together determine the final redox state at the β-carbon of each chain extension unit, as well as *C*-, *O*- and *N*-methyltransferases. The corresponding essential domains in NRPS systems are the adenylation (A), condensation (C) (or heterocyclization (HC)) and peptidyl carrier protein (PCP), while the complement of specialized processing enzymes can include epimerase (E), *N*- and *C*-methyltransferase and oxidase (Ox) activities. In most cases, the finished products are released from the carrier proteins by dedicated thioesterase (TE) domains, located at the C-terminal ends of the assembly lines, either as free acids, or more commonly, macrolactones. The assembly line analogy extends further, as the modules are ordered within the multienzymes in the order in which they act. In fact, the co-linearity between the genetic organization and the sequence of enzymatic transformations in many systems is so strong, that multiple features of the product structures can be predicted with confidence from inspection of the gene sequences alone.

Almost from their discovery, myxobacterial PKS, NRPS and mixed systems have challenged this 'textbook' logic of modular megasynthase operation.[92,135] To date, the complete sequences of two NRPS, two PKS, and 16 mixed PKS-NRPS clusters have been reported, and each new system revealed surprising deviations from the established rules. For example, one of the classical (and conve-

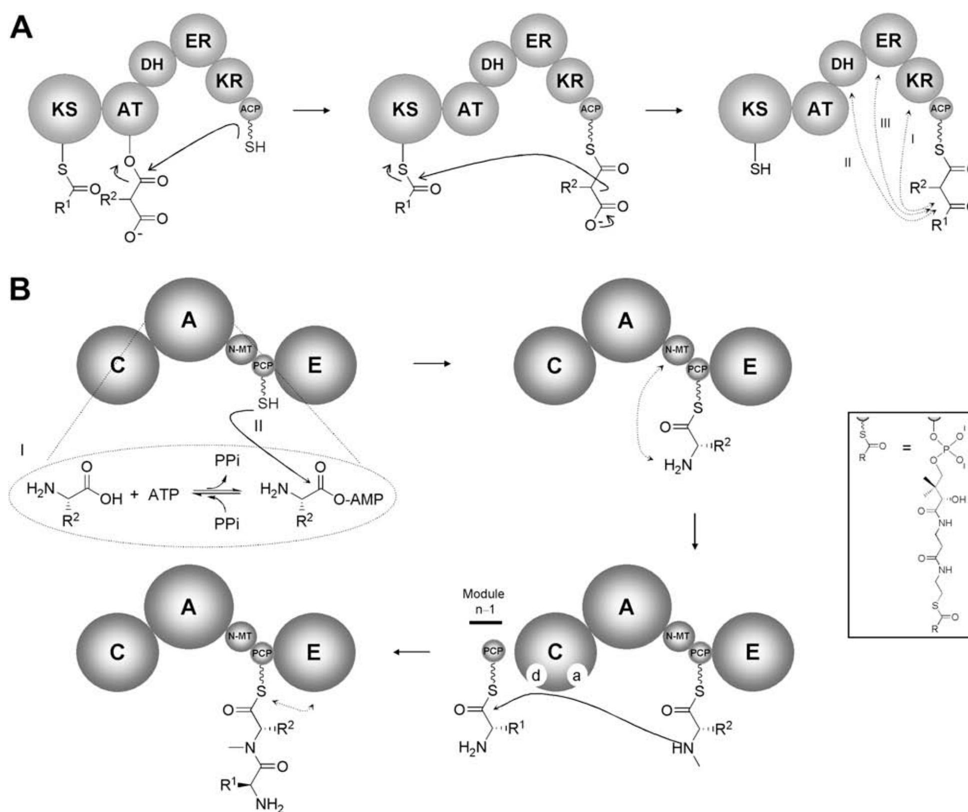

Figure 6. Assembly line biosynthesis of polyketide (PK) and non-ribosomal peptide (NRP) natural products. (A) Set of reactions which occurs in a typical polyketide synthase (PKS) module. Following selection of an extender unit (typically malonate or methylmalonate) by the acyltransferase (AT) domain, the building block is transferred to the acyl carrier protein (ACP). The ketosynthase (KS) then catalyzes a thioclaisen condensation between the growing chain tethered to its active site thiol, and the extender unit attached to the ACP. If reductive domains are present (here ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER)), the resulting β-keto thioester undergoes redox adjustment. (B) Set of reactions which occurs in a typical non-ribosomal peptide synthetase (NRPS) module. An amino acid is selected and activated as its adenylate by an adenylation (A) domain, followed by transfer to the peptidyl carrier protein (PCP). Optional *N*-methylation can then occur catalyzed by the *N*-methyltransferase (N-MT) followed by condensation (C)-domain catalyzed peptide bond formation. If an epimerase (E) domain is present, it is likely to act following chain extension. Domains are shown approximately to scale. Inset is the prosthetic phosphopantetheine attached to the carrier protein domains.

nient) features of biosynthetic gene clusters in other microbes is that the full complement of genes is clustered into one location in the chromosome. In myxobacteria, however, multiple clusters have been discovered in which essential functions are apparently 'missing', and therefore presumed to be located elsewhere in the genome. This is the case with the chivosazol,[96] disorazol[136] and tubulysin[137] systems. Such a 'split-cluster' organization raises the intriguing, and as yet unanswered question of how all of the genes are co-ordinatively regulated.

In many myxobacterial PKS and mixed systems, biosynthesis gets off to an unusual start. When a polyketide building block is used to initiate biosynthesis in *Streptomyces*, it is very commonly acetate or propionate; selection and activation of the starter unit are performed by an AT-ACP loading didomain, or more commonly, a KS^Q-AT-ACP tridomain (the KS^Q catalyzes decarboxylation of a malonate or methylmalonate unit, to yield respectively acetate or propionate).[138] In contrast, biosynthesis of myxobacterial compounds often commences with moieties other than acetate or propionate, including benzoate,[139] isovalerate,[100] isobutyrate,[9,140] 2-methylbutyrate,[140] dehydro-isobutyrate,[102] 3-hydroxyvalerate,[12] pipecolic acid,[137] and polyunsaturated fatty acids.[118,119] Correspondingly, the domains which incorporate these more unusual starter units, are themselves atypical (although divergent architectures are also present in modules which select acetate); in fact, in none of the clusters sequenced to date is there a 'typical' loading module. Several loading modules (e.g., those from the ambruticin/jerangolid[10] and melithiazol[102]/cystothiazole[40] systems) appear to be standard in that they incorporate a KS-AT-ACP tridomain unit. However, the KS domain contains an active site Cys instead of the Glu associated with decarboxylating KSs, and is therefore expected to be active for chain extension. The epothilone PKS includes a unique KS^Y-AT-ER-ACP loading module;[141,142] the KS^Y has been proposed to function like a KS^Q, providing an acetate unit from malonate by decarboxylation, but alternatively the AT domain may activate acetate directly.[143] Another common variation is a mixed loading/first extension module, with a domain order of ACP-KS-AT_1-AT_2-(DH-ER)-KR-ACP.[9,11,100,109,139,140,144] Studies on the ATs in soraphen biosynthesis have demonstrated that the first AT is responsible for starter unit selection, while the second delivers the extender unit to the ACP of the first module.[139] The loading module of the chivosazol PKS is also puzzling, as it consists only of an ACP domain.[96] However, the presence of a discrete GCN5-related acetyltransferase (GNAT) domain in the cluster might explain the origin of the acetate unit (see discussion of myxovirescin biosynthesis, in Section 6). The disorazol system is even more peculiar, as a loading module is completely missing; the basis for selection of the acetate starter unit is thus unclear.[136] Finally, chain extension in the myxochromide pathways is initiated by the iterative operation of a PKS module, which produces polyunsaturated fatty acids of varying length.[118]

The organization and domain complement of myxobacterial modules also deviates significantly from the textbook models (e.g., KS-AT-(DH-ER-KR)-ACP in the case of PKS modules, and C-A-PCP, in the case of NRPS modules). For example, many modules appear to be missing domains which are required for the

biosynthesis. One of the most common variants is PKS modules which lack AT domains. This feature is common to all '*trans*-AT' PKSs, a growing class of PKS systems from a range of organisms.[145,146] In these cases, the acyl transferase function is provided instead by a discrete AT domain or domains, which act iteratively to load a common extender unit (typically malonate) onto all of the ACP domains present in the megasynthases.[147] The basis for association between the *trans* AT and each PKS module remains to be elucidated. DH functions appear to be missing in modules within the aurafuron,[9] chivosazol,[96] epothilone,[141,142] myxalamid,[140] stigmatellin,[109] and spirangien[11] systems. A potentially general mechanism by which this activity is complemented has recently been elucidated for epothilone biosynthesis: following chain extension in the DH-deficient module (module 4), the intermediate is passed to the ACP of the subsequent module where it can interact with a functional DH domain, before being transferred upstream to the module 5 KS domain to resume chain extension.[148]

In contrast, some modules harbor catalytic functions which appear to be active, but which are not required for the biosynthesis

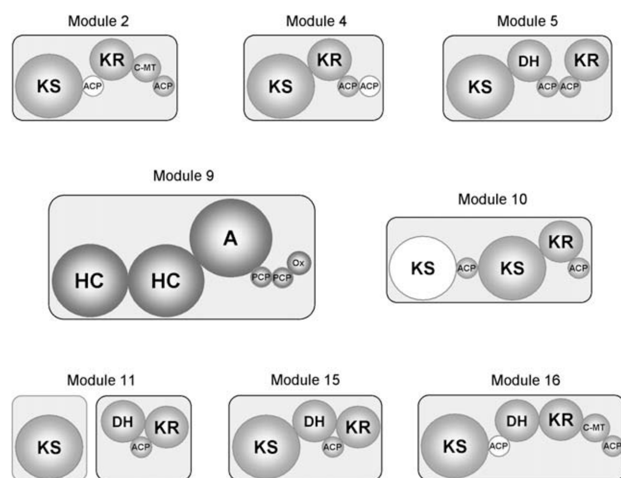

Figure 7. Non-classical modular architectures found within the chivosazol mixed PKS-NRPS assembly line. Domains expected to be inactive are shown in white. *Abbreviations:* C-MT, *C*-methyltransferase; HC, heterocyclization; Ox, oxidase.

(e.g., extra reductive activities[9,12,141,142]), while many systems contain the remnants of inactive domains. Some modules incorporate multiple copies of the same domain (e.g., ACPs or HCs),[10,96,136] while in others, domains are present in an unusual order.[10,96,137] This phenomenon is most pronounced in the chivosazol cluster,[96] in which eight PKS modules exhibit different domain compositions (Fig. 7). It is presently not obvious how these variant domain arrangements can be accommodated in a shared architecture for PKS modules. Finally, the modules themselves show atypical behavior: modules can be intentionally skipped (myxochromide S,[119] chivosazol,[96] and disorazol[136]) or iterate deliberately (aurafuron,[9] the myxochromides,[118,119] stigmatellin[109]), while others are split between two multienzymes (e.g., ambruticin,[10] chivosazol,[96] disorazol,[136] myxalamid,[140] and myxochelin[85]). The basis for reconstitution of split module function remains unknown, as does the mechanism controlling the number of times a particular module operates (none, once or multiple times).

In classic PKS and mixed systems, chain termination is typically accomplished by a thioesterase domain. While this is the case with many myxobacterial assembly lines, a number of unusual release mechanisms are also operative. For example, biosynthesis of the bisthiazoles myxothiazol **8** and melithiazol **9** occurs with an extra chain extension step, catalyzed by subunits MelG and MtaG, respectively (Fig. 8). The added glycine is then hydroxylated by an integral monooxygenase (Mox) domain to yield an unstable intermediate, which can then decompose (catalytically or spontaneously) to yield the free amide. In the case of melithiazol **9**, the amide functionality is subsequently hydrolyzed to the carboxylic acid by enzyme MelJ, and then esterified to yield the methyl ester by MelK.[149] The polyketide stigmatellin contains a rare aromatic chromone ring, within an otherwise reduced skeleton. Cyclization is likely catalyzed by the terminal cyclase (Cy) domain of the assembly line, although this hypothesis awaits experimental verification.[109] Finally, the aurafuron PKS lacks a thioesterase or any other termination function within the last subunit. Disconnection from the PKS may therefore occur spontaneously, or by oxidative cleavage following additional processing by post-PKS enzymes.[9] Efforts to understand these alternative release mechanisms are currently underway in the laboratory.

Myxobacterial secondary metabolite family members often differ from each other through variations in functionalities displayed on the core of the molecules (although post-assembly line tailoring

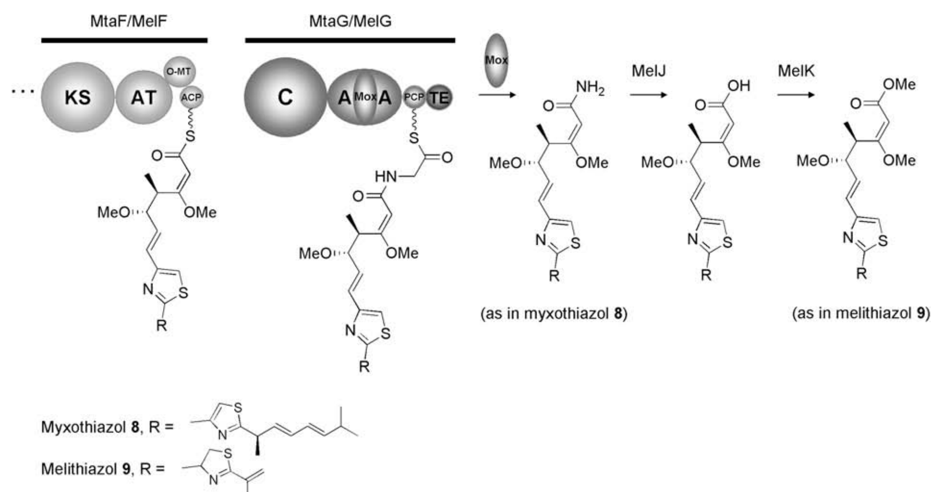

Figure 8. Model for biosynthesis of the amide and methyl ester functionalities in myxothiazol **8** and melithiazol **9**. The intermediates shown are generated by the first four subunits in the pathway (MtaB–MtaE/MelB–MelE, respectively), followed by chain extension on MtaF/MelF. The thioester functionalities are then transformed into amides by extension with glycine and subsequent oxidative cleavage, catalyzed by MtaG/MelG. In the melithiazol pathway the amide moiety is hydrolyzed to the free acid by MelJ, and then methylated by MelK. Abbreviation: Mox, mono-oxidase.

reactions also enhance structural diversity). Such differences are very likely to arise from alternative operation of the assembly line proteins. For example, the presence of a keto, hydroxyl, or unsaturation at a particular center can be explained by different levels of reductive processing within the corresponding module. Similarly, variation in starter units, or to the extent of methyl branching at specific centers, can be accounted for by broad specificity AT domains. Equally, promiscuous A domains in NRPS modules can lead to incorporation of several different amino acids at a specific position in the metabolites. Thus one relatively common feature of myxobacterial assembly lines, in contrast to those from other species, appears to be the highly imperfect nature of the biosynthesis.

6. Myxovirescin biosynthesis in *M. xanthus* DK1622: a case study

Biosynthesis of the myxovirescins (or TA antibiotics) exemplifies many diverse aspects of myxobacterial secondary metabolism, and so it is worthwhile to analyze the pathway in detail here (Figs. 9 and 10). The myxovirescins are a large family of mixed PK-NRP compounds known exclusively from the genus *Myxococcus* (including *M. xanthus* ER-15 and *M. virescens* Mxv48)[122,150–155] and recently identified in the model genome strain *M. xanthus* DK1622.[12] The compounds exhibit wide-spectrum activity against Gram-negative bacteria, and adhere strongly to dental tissues, suggesting their utility for the treatment of periodontal disease.[156–158] More than 30 myxovirescin analogues have been discovered to date in *M. virescens*. Variation relative to myxovirescin A **18** (Fig. 10), the major metabolite, occurs at multiple sites, including the level of reduction at C20, C24 and C26, and across the C22–C23 bond.[159] Some analogues incorporate an unusual ethyl branch at C16, while in others it is a methyl group, while the methyl branch at C12 is tailored to varying extents (to a hydroxyl, a methoxy, or a carboxylic acid). Remarkably, analogues have been detected which are both smaller and larger than myxovirescin A, by one chain extension unit. Strain DK1622 produces only two myxovirescins at detectable levels, myxovirescin A and C, which differ in the functionality present at C20 (either a ketone or a methylene).

The entire gene cluster has recently been cloned and sequenced, and found to occupy approximately 83 kbp on the *M. xanthus* genome. The locus contains four ORFs encoding type I PKS subunits (*taI*, *taL*, *taO* and *taP*), and one gene encoding a hybrid PKS-NRPS (*ta-1*) (Fig. 9). These gigantic genes are flanked on both sides by various ORFs with similarity to the type II enzymes of fatty acid biosynthesis. The closest homologues to myxovirescin genes are found in the clusters for leinamycin (from *Streptomyces atroolivaceus* S-140[160]), mupirocin (from *Pseudomonas fluorescens* NCIMB 10586[161]) and pederin (from an uncultured bacterial symbiont of the beetle *Paederus fuscipes*[162]). Notably, all of these systems belong to the class of 'AT-less' PKSs. Analysis of the gene cluster both by insertional mutagenesis in DK1622,[12,163,164] and by expression of recombinant proteins in vitro,[165] has elucidated many aspects of the biosynthesis.

As in many mixed PKS-NRPS systems from myxobacteria, initiation of myxovirescin biosynthesis occurs by an unusual mechanism, which has been only incompletely deciphered. Feeding

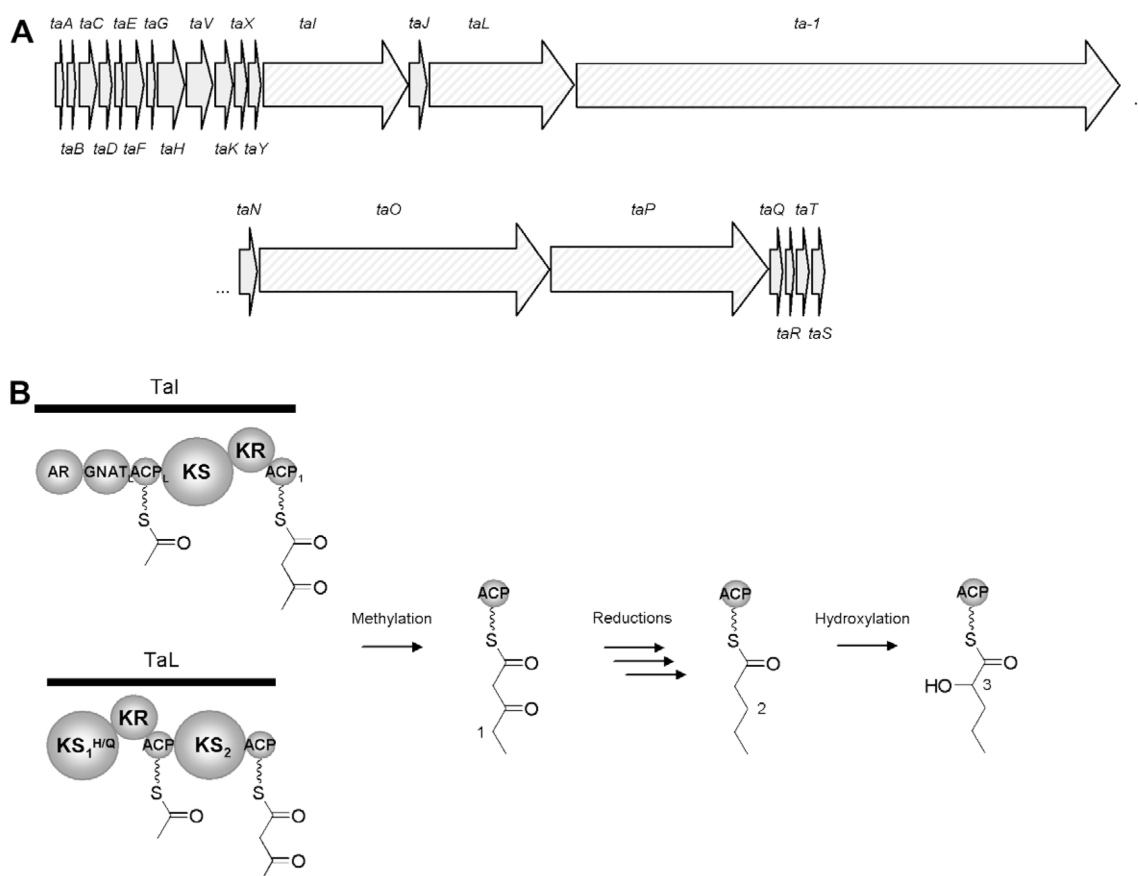

Figure 9. (A) Organization of the 82.8 kbp myxovirescin biosynthetic gene cluster. The PKS/NRPS genes are shown in grey hatching. (B) Two possible routes to generate the myxovirescin starter unit. Both TaI and TaL may catalyze the condensation of acetate with malonyl-ACP to yield acetoacetyl-ACP. The acetoacetyl-ACP then undergoes methylation at C1 (numbered according to myxovirescin), reduction at C2, and finally hydroxylation at C3 to yield the 3-hydroxylvaleryl-ACP moiety. Abbreviations: AR, adaptor region; GNAT, GCN5-related *N*-acetyltransferase; $KS^{H/Q}$, ketosynthase containing an active site His to Glu substitution.

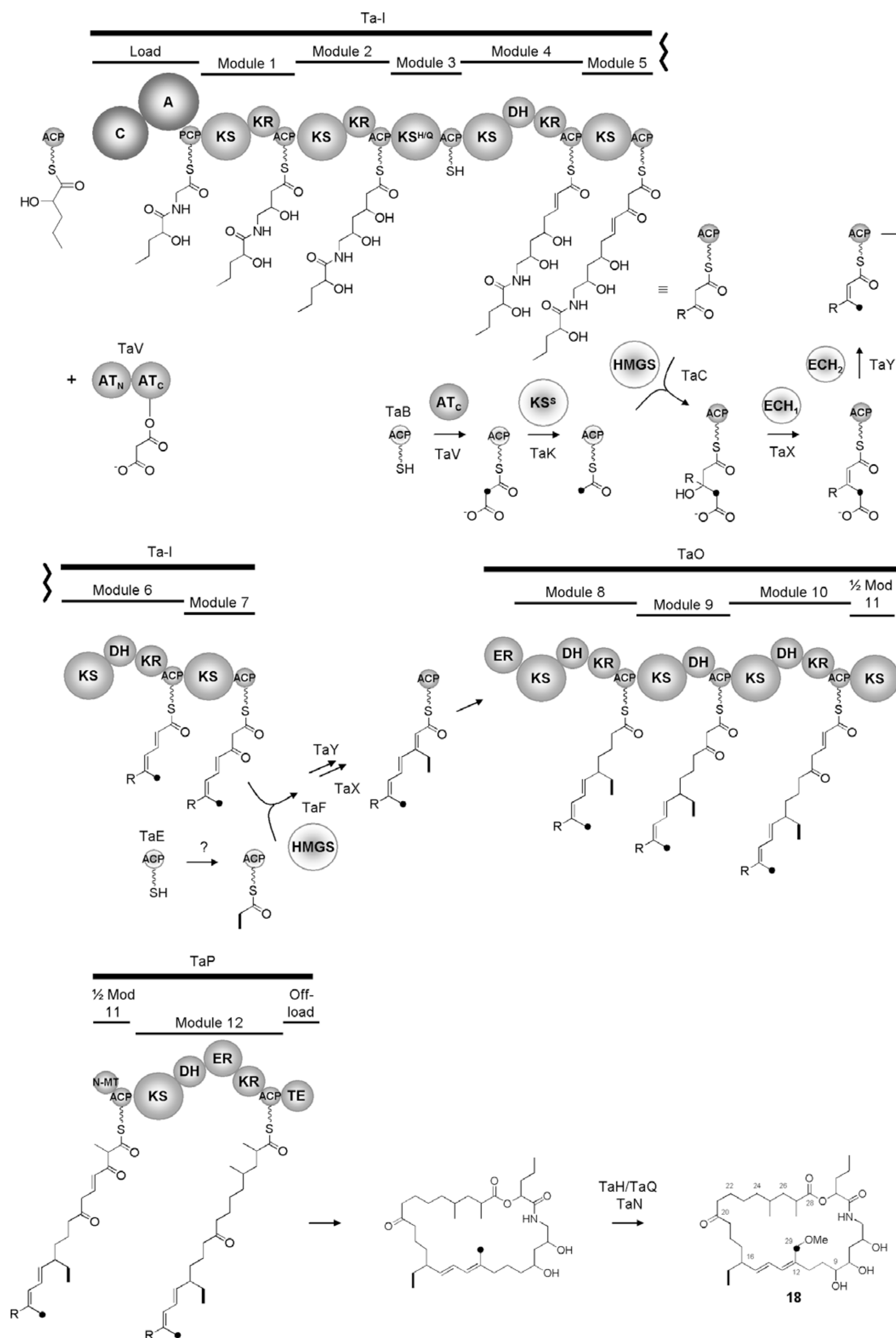

Figure 10. Proposed biosynthetic pathway to myxovirescin A, based on evidence obtained in vitro and in vivo. The lone ACP carrying the starter unit represents the terminal domain of either TaI or TaL (see Fig. 9). The starter unit is then extended by modules of subunit Ta-I, although module 4 is likely to be inactive due to a KS active site mutation. Following elongation by module 5, the biosynthesis switches to HMGS biosynthetic logic, in order to introduce a β-methyl branch. This chemistry involves five enzymes, AT_c, KS^S, HMGS, ECH_1 and ECH_2. Chain extension then resumes with modules 6 and 7, followed by installation of the β-ethyl group, again using HMGS chemistry. Construction of the chain then finishes with subunits TaO and TaP. The resulting enzyme-free intermediate is further transformed to myxovirescin A by formation of a methoxy at C-29 (catalyzed by TaH/TaQ), and hydroxylation at C-9, accomplished by TaN. Abbreviations: AT_N, N-terminal AT of the didomain AT, TaV; AT_C, C-terminal AT of the didomain AT, TaV; KS^S, decarboxylating ketosynthase containing an active site Cys to Ser substitution; HMGS, 3-hydroxy-3-methylglutaryl synthase; ECH, enoyl-CoA hydratase; TE, thioesterase.

studies have suggested that the starter unit is constructed by the condensation of an acetate and a malonate unit, followed by methylation of the C1 carbon with an *S*-adenosylmethionine (SAM)-derived methyl group, reduction at C2, and hydroxylation at C3.[12] Insight into one likely origin of the acetate unit has recently provided by studies of the mixed PKS-NRPS responsible for biosynthesis of curacin A in the marine cyanobacterium *Lyngbya majuscula*.[166] CurA, the first multienzyme of the pathway, contains

a loading module comprised of an N-terminal adaptor region (AR), a GCN5-related *N*-acetyltransferase (GNAT) domain, and an ACP domain. GNAT is a superfamily of *N*-acyltranferase enzymes which typically catalyze direct acyl transfer to primary amines.[167] Studies using recombinant curacin AR-GNAT$_L$-ACP$_L$ have shown, however, that the CurA GNAT domain catalyzes decarboxylation of malonyl-CoA to acetyl-CoA, followed by AR-assisted transfer of the acetate group to the adjacent ACP, an unprecedented reaction for this group of enzymes. Catalysis by an AR-GNAT didomain therefore represents a third enzymatic strategy for PKS chain initiation.

Notably, the same tridomain AR-GNAT-ACP architecture is present in TaI of the myxovirescin pathway. Thus one possible source of the starter unit is biosynthesis by bimodular TaI (Fig. 9). Following formation of acetyl-ACP$_L$ by the GNAT domain, condensation with a malonate unit attached to ACP$_1$ would afford acetoacetyl-ACP$_1$. Methylation at C1 (possibly by the radical SAM methyltransferase TaS[168,169]), reduction by KR$_1$ and C3 hydroxylation (by the putative oxygenase TaJ), would yield the proposed hydroxyvaleryl-ACP starter unit. However, the cluster contains a second putative PKS initiation module, TaL, which exhibits a KS-KR-ACP-KS-ACP architecture. One of the conserved His residues in KS$_1$ is substituted by Gln, and so the KS is likely to be inactive as a condensation catalyst. However, KS$_2$ could in principle catalyze condensation between acetate and malonate tethered to its flanking ACPs to yield acetoacetyl-ACP. Indeed, inactivation of *taI* and *taL* separately and in combination, demonstrated that either subunit is adequate to supply low levels of starter unit, but that both are required to achieve wild type levels of myxovirescin.[12] A detailed explanation of this finding awaits further analysis.

Regardless of the origin of the starter unit, it is then condensed with glycine attached to the PCP domain of Ta-1 (Fig. 10). Chain extension with malonate is then performed successively by modules 1 and 2 of Ta-1. As anticipated from the phylogenetic analysis, myxovirescin is an AT-less PKS. Studies in vitro of recombinant TaV$_C$ (the C-terminal domain of the unusual didomain AT, TaV) suggest that it transfers malonate to each of the ACP domains in the assembly line.[170] In-frame deletion of *taV* abolished myxovirescin biosynthesis, confirming the involvement of its gene product TaV in the pathway.[12] Analysis of the myxovirescin structure strongly suggests that condensation by module 3 is skipped. Consistent with this expectation, the KS domain of module 3 of Ta-1 contains a His to Gln mutation, and so is likely to be defective in decarboxylation. However, KS$_3$ may still aid in transfer of the chain extension intermediate from ACP$_2$ to KS$_4$ via ACP$_3$; alternatively, the reaction may occur directly through ACP$_3$, or module 3 may be bypassed entirely (direct ACP$_2$ to ACP$_4$ transacylation). Modules 4 and 5 then carry out chain extension (resulting in a β-keto functionality), at which point the pathway switches to 3-hydroxyl-3-methylglutaryl-CoA synthase (HMGS) biosynthetic logic in order to introduce the first of two β-alkyl branches, a methyl group at C12.[163,164,170] A β-ethyl group is added following chain extension by modules 6 and 7.

In bacillaene biosynthesis,[170] addition of a nucleophilic methyl group has been shown to require a cassette of five proteins, comprising an HMGS, a decarboxylase-type KS domain (which incorporates an active site Cys to Ser substitution), a discrete ACP, and two enoyl-CoA hydratase homologs (ECHs). Support for the assigned functions has been provided by analysis of several other gene clusters, including those for curacin,[171,172] jamaicamide,[173] mupirocin[174] and pederin/onamide.[175] As myxovirescin incorporates two alkyl branches, in principle, the cluster could have contained two complete β-alkylation cassettes. However, while two HMGS homologues (TaC and TaF) and two putative ACP domains (TaB and TaE) are present, the remaining catalysts are found only in single copy (the KS^S TaK, and the ECH enzymes, TaX and TaY), suggesting functional redundancy in the pathway.[12]

To elucidate the precise division of labor, all of the genes have been selectively inactivated (both singly and in combination),[12,163,164] and each protein studied in recombinant form in vitro.[165] Together, these studies have revealed significant insight into the functional elaboration of the myxovirescin backbone (Fig. 10). In the case of the C12 methyl group, ACP TaB is malonated by action of the AT domain TaV$_C$,[170] and then the malonyl-*S*-TaB is decarboxylated by the KS^S TaK, to yield acetyl-*S*-TaB. HMGS homologue TaC then catalyzes nucleophilic attack of the acetyl enolate on to the β-ketothioester of the module 5 intermediate. TaX dehydrates the HMG derivative (either α,β or β,γ with respect to the thioester), followed by TaY catalysis which results in overall decarboxylation to give the Δ^2 β-methylated product. The final two-step conversion of the methyl to the methoxy functionality occurs through oxidation by the cytochrome P450 TaH to the hydroxyl, followed by methylation by the SAM-dependent methyltransferase TaQ, likely after the chain extension intermediate is released from the assembly line.[12,164]

The ethyl group is installed by a broadly similar mechanism. The functional ACP/HMGS pair in this case is formed by TaE and TaF; like *taB* and *taC*, *taE* and *taF* are translationally coupled. By analogy to methyl group formation, the propionate unit was predicted to arise from TaK-catalyzed decarboxylation of methylmalonyl-*S*-TaE.[12,170] However, this mechanism is likely to be wrong, as TaK does not show any activity towards this substrate, at least in vitro.[170] Therefore, either a different enzyme catalyzes decarboxylation of methylmalonyl-*S*-TaE, or propionate is loaded directly onto TaE by a discrete AT domain. Surprisingly, neither of the TaV AT domains catalyzes propionyl transfer to TaE at least in vitro, suggesting that if another *trans* AT exists, it is located outside the sequenced region. The remainder of the pathway follows the methyl group precedent, with TaF-catalyzed condensation, followed by dehydration and decarboxylation accomplished by TaX and TaY, respectively. An additional important conclusion arising from the inactivation studies is that the functions of the two ACP/HMGS pairs are not mutually complementary. The TaB/TaC pair can rescue myxovirescin production in the absence of functional TaE or TaF, but reverse complementation of Δ*taB* and Δ*taC* strains was not observed. The analog (called myxovirescin ΔF) observed in the Δ*taE* and Δ*taF* mutants incorporates a methyl group instead of an ethyl group at C16,[163] the same structural variation found in naturally occurring myxovirescins. The fact that TaB/TaC can substitute for TaE/TaF implies a level of flexibility in the docking interaction between the ACP/HMGS complex and module 7. However, the precise molecular determinants for this protein–protein recognition event are unknown at present.

Following addition of the β-branches, the system reverts to PKS logic with chain extension by module 8 (Fig. 10). Interestingly, the ER required to complete the set of reductive reactions in this cycle is located upstream of KS$_8$. The unusual position of this domain may allow it to reduce the β-ethylation intermediate generated by module 7, as well as to participate in the reductive cycle in module 8.[12] Chain extension then proceeds with modules 9 and 10 of TaO, where module 9 is notable for the presence of an active, but superfluous DH domain. Module 11 is split between two proteins, with the KS domain located on TaO, and the MT-ACP remainder of the module on multienzyme TaP; rigorous re-sequencing of this region has confirmed that the split module is genetically encoded. Both modules 10 and 11 lack the full complement of reductive domains required to generate the functionality observed in the major myxovirescin metabolites. These activities may instead be provided by the twelfth and final chain extension module, which incorporates a full reductive 'loop'. Again, the ER occupies a non-standard position, between the ACP and TE domains, which may allow it to operate independently of the DH and KR activities. The requirement for simultaneous reduction at multiple sites on the

intermediate (C26, C24 and across the C23–C22 double bond) during this condensation cycle may explain the presence of myxovirescin analogues which show varying levels of processing at these positions, as well as at C20. Alternatively, the module 9 and 10 intermediates skip from their respective modules to module 12 and back, in order to achieve the observed reduction levels, before resuming the normal mode of chain extension. Finally, the intermediate is released as a macrolactone using an internal hydroxyl nucleophile, by the C-terminal TE domain. In addition to methoxy formation at C29 (as described earlier), post-assembly line hydroxylation takes place at C9, likely catalyzed by the TaN dioxygenase.

This analysis shows that myxovirescin biosynthesis incorporates many of the divergent features noted for myxobacterial systems: assembly occurs on a hybrid PKS-NRPS system, incorporating a chimaeric multienzyme Ta-1; construction of the starter unit involves a rare GNAT domain, and possibly two independent modules; extender units are provided by a *trans*-acting AT domain, located in a rare AT didomain; module 3 is a skipped module; a superfluous DH domain is present in module 9; reductive domains are missing from modules 10 and 11, and are likely complemented by activities in module 12; the ER domains of modules 8 and 12 occupy unusual locations; and the biosynthetic logic switches to HMGS machinery and back again two times. Finally, the imperfect operation of many of these machineries likely accounts for the generation in at least several strains, of a large metabolite family. This analysis clearly shows that many features of the structure could not have been predicted directly from analysis of the cluster sequence, illustrating the challenges of 'genome mining' in myxobacterial strains.

7. Future strategies for exploiting myxobacterial secondary metabolism

Both the ongoing genome sequencing efforts and metabolic profiling of myxobacterial strains, suggest that the depth of myxobacterial secondary metabolism is far greater than previously appreciated. Therefore, future efforts in this field must focus on 'mining' the genomes of both terrestrial and marine myxobacteria for novel compounds.[116,176] A number of strategies can be employed both to identify metabolites made under standard laboratory conditions (particularly those which are produced in trace amounts), as well as to 'awaken' clusters which have to date remained silent. For example, the culture conditions (including media composition, culture vessel, temperature, pH, aeration rate, presence of enzyme inhibitors, etc.) of the producing organism can be varied systematically. This approach, called 'one strain-many compounds' (OSMAC), has been applied to both fungi and Actinomycetes, resulting in each case in the isolation of new metabolites.[117] Less conventional and as yet untested methods for inducing secondary metabolism in myxobacteria, might include introduction into the cultures of a competing microorganism ('bacterial challenge'),[26] as well as cytotoxic compounds.[177]

When genetic information is available for PKS and NRPS systems, predictions can be made about likely building blocks, as well as elements of the final structure (although this is often less straightforward in myxobacterial systems[92]). Specific precursors can then be supplied in order to increase compound yields, or the growth conditions can be tailored to favor production of the metabolite.[176] Even the reasonable expectation that a particular functional group will be present (e.g., a chromophore) can aid during compound identification by standard analytical methodologies, such as HPLC-diode array MS. In parallel, the producing strain can be manipulated genetically. For example, biosynthesis from a known, but cryptic gene cluster may be triggered by over-expression of a specific activator gene, as demonstrated recently with the fungus *Aspergillus nidulans*.[178] The first putative regulatory element for a myxobacterial metabolite was identified by random transposon mutagenesis in the myxobacterium *Cystobacter fuscus* Cb f17. This approach revealed both the biosynthetic gene cluster for the polyketide stigmatellin in the strain, and its two-component regulator, StiR.[179] Subsequently, biomagnetic bead separation of promoter-binding proteins coupled with gene inactivation, were used to identify the protein ChiR, a positive regulator of chivosazol biosynthesis in *Sorangium cellulosum* So ce56. Over-expression of *chiR* resulted in as much as 5-fold overproduction of chivosazol.[180] Alternatively, the cluster can be deliberately inactivated, and resulting mutants screened for the absence of specific metabolites relative to the wild type strain. This strategy was used successfully to identify the myxochelins,[85] myxochromides[118] and aurafurones.[9]

In cases of orphan compounds, random transposon mutagenesis is a proven method for obtaining the corresponding genetic information. Recovery of the transposon and flanking regions of the genome from metabolite non-producers (or less often, over-producers),[181] can facilitate the design of specific probes for the cluster of interest within a cosmid library of the strain. This approach recently yielded the ambruticin/jerangolid,[10] aurachin,[14] disorazol[136,182] and tubulysin[137] gene clusters. Alternatively, highly conserved PKS or NRPS domains (e.g., KS, HC, A) expected to participate in the pathway can be amplified from genomic DNA using degenerate primers, and then individual sequences used to inactivate their respective clusters. The obtained products can be employed directly to screen a cosmid library of the strain (as with epothilone[141] and spirangien[11]), or cloned into gene disruption plasmids, and pre-evaluated for their ability to abolish production of the target metabolites (as with chivosazol[96]). In future, the clusters corresponding to known compounds may be identified routinely by shot-gun genome sequencing, as demonstrated recently for the phosphoglycolipid moenomycin A.[183]

If the producing strain is refractory towards manipulation, one powerful alternative approach for investigating both assigned and orphan gene clusters is heterologous expression in more genetically and fermentation friendly hosts. This technology has been reviewed in detail elsewhere,[184] so the strategy is only briefly summarized here. Typically, a target gene cluster is co-expressed in a suitable host from several plasmids, or pre-assembled into a single construct in *E. coli* from one or more cosmids or BACS. The 'stitching' process for cluster reconstruction, once a considerable barrier to heterologous expression, has been significantly enabled by Red/ET recombination technology.[185] In future, advances in the total synthesis of DNA may allow for gene sets of any size to be assembled from scratch, in codon optimized form,[186] obviating the need for genetic engineering. Hosts under development for heterologous expression of myxobacterial clusters include *Myxococcus xanthus* and several *Pseudomonas* strains. As a myxobacterium, *M. xanthus* shares its codon usage and physiology with many other species, and gene cluster-specific promoters are also likely to be active. Furthermore post-translation phosphopantetheinylation of PKS and NRPS proteins should be very efficient, and the strains are likely to contain the required metabolic precursors. The main advantages of *Pseudomonads* over *M. xanthus*, is that growth rates are on par with *E. coli*, and plasmids harboring inducible promoters are available. However, not all myxobacterial regulatory elements are recognized in *Pseudomonas* strains, often necessitating additional engineering steps. Proof of principle is now well established, with a number of clusters expressed successfully: epothilone in *E. coli*,[142] *Streptomyces coelicolor*[187] and *M. xanthus*,[188] soraphen in *Streptomyces lividans*,[190] myxochromide S **19** in *Pseudomonas putida* (Fig. 11),[189,191] flaviolin in three *Pseudomonas* strains,[130] and myxothiazol in both *M. xanthus*[192] and *P. putida*.[193] Nonetheless, it will be important to continue to identify additional heterologous hosts, as no single 'super host' strain is likely to serve in all cases.

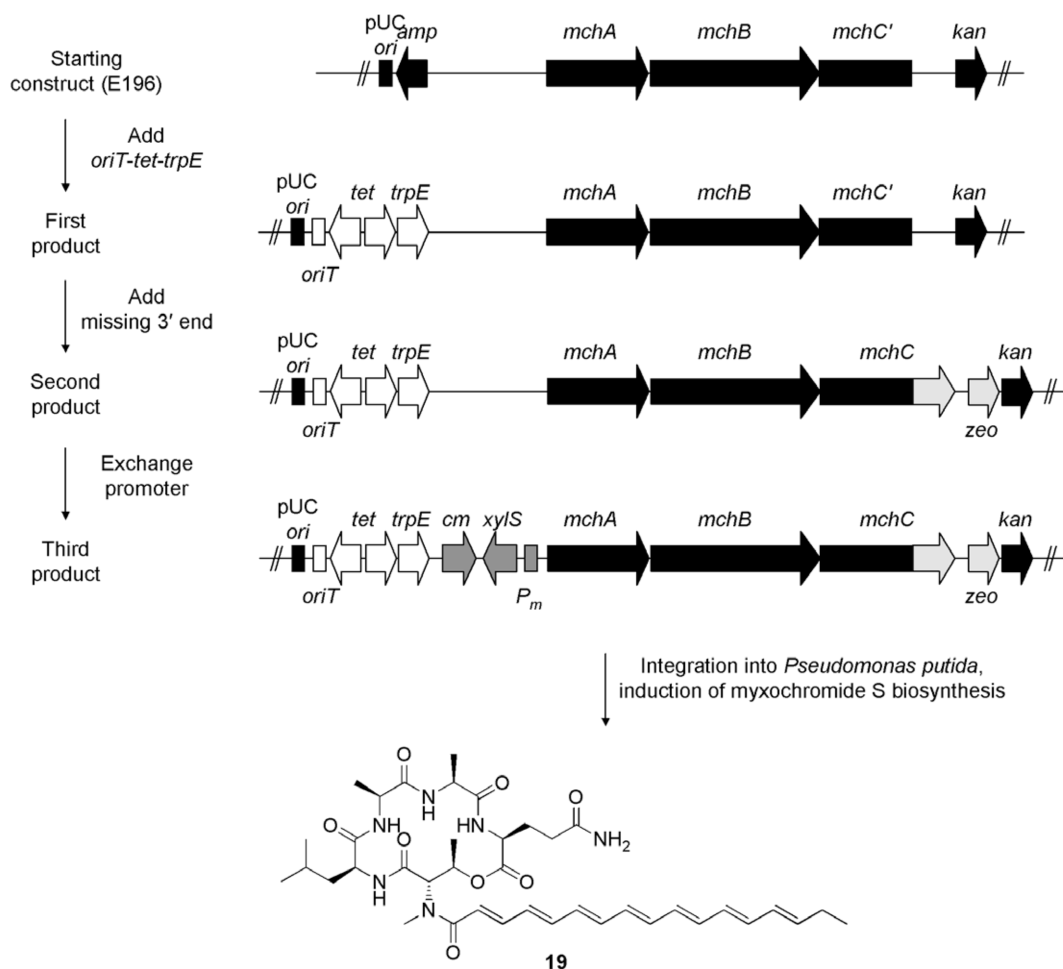

Figure 11. DNA engineering strategy employed to reconstitute the pathway to myxochromide S in the heterologous host *Pseudomonas putida*. Maps of the starting cosmid and the Red/ET recombination constructs are shown. First, the starting construct (cosmid E196) was modified by single-step insertion of the origin of transfer (*oriT*) to enable conjugation, the tetracycline resistance gene (*tet*) for selection in *P. putida*, and a DNA fragment (*trpE*) from the chromosome of *P. putida* to enable integration of the construct into the genome by homologous recombination (first product). The missing part of gene *mchC* (encoding the TE domain) was then added (second product). Finally, the toluic acid inducible P_m promoter was inserted in front of the first gene of the myxochromide S cluster (third product). Figure adapted from Ref.191.

In parallel, efforts must continue towards deciphering the unusual features of assembly line biosynthesis in myxobacteria. Just as 'the exception proves the rule', these divergent systems have important implications for overall models of modular PKS and NRPS architecture and mechanism, and are particularly relevant for understanding hybrid synthetases. Studies are likely to focus on PKS and NRPS domains which catalyze rare or novel chemistries, as well as unique post-assembly line tailoring activities. PKS and NRPS modules which exhibit non-standard domain composition and ordering or iterative behavior are very attractive targets for crystallographic studies, alongside more 'standard' modules derived from *Streptomyces* or *Bacillus* assembly lines. In parallel, research should continue to explore the protein-protein interactions which underlie the pathways, particularly in variant or split modules. Together, such studies should ultimately enable attempts to rationally engineer myxobacterial biosynthetic machineries towards to the production of novel metabolites (so-called 'combinatorial biosynthesis'[99]).

8. Conclusions

Myxobacteria, already known to produce some 5% of known bacterial natural products,[3] have only begun to reveal the depth and diversity of their secondary metabolism. However, the advent of the myxobacterial 'genomic era' (with four genomes published to date, (refs.[125,194]; http://xbase.bham.ac.uk/genome.pl?id=1644; http://cmr.tigr.org/cgi-bin/CMR/GenomePage.cgi?org=ntad01) and a fifth nearing completion[8]), as well as enormous progress in the isolation, fermentation and genetic manipulation of many myxobacterial strains, look set to revolutionize the study of myxobacterial biosynthesis in the near future. Undoubtedly, newly-discovered strains and their metabolites will continue to unveil novel chemistries and enzymatic mechanisms, as well as to challenge 'textbook' thinking about modular multienzyme assemblies. In parallel, it is likely that additional myxobacterial metabolites (or their close derivatives) will reach the clinic. The future of myxobacterial natural products research looks very promising, indeed.

9. Notes Added in Proof

Ebright and colleagues[195] have recently demonstrated that corallopyronin (Cor) and ripostatin (Rip) interact with the 'switch region' of RNA polymerase (RNAP). This region of the enzyme is distant from the targets of previously characterized RNAP inhibitors, explaining the lack of cross-resistance to Cor and Rip. A radical SAM enzyme has recently been demonstrated to carry out the methylation of an unactivated methyl center in the biosynthesis of the myxobacterial antibiotic chondrochloren B.[196] This finding

supports our proposal that similar chemistry is employed to produce the starter unit in myxovirescin biosynthesis. Finally, Fu *et al.* have recently shown that transposons can be exploited for the efficient introduction of secondary metabolite pathways into several heterologous hosts.[190] This technique is likely in future to become the method of choice for delivery of large transgenes.

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