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REVIEW

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Analysis of myxobacterial secondary metabolism goes molecular

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Abstract During the last 20 years myxobacteria have made their way from highly exotic organisms to one of the major sources of microbial secondary metabolites besides actinomycetes and fungi. The pharmaceutical interest in these peculiar prokaryotes lies in their ability to produce a variety of structurally unique compounds and/or metabolites with rare biological activities. This review deals with the recent progress toward a better understanding of the biology, the genetics, the biochemistry and the regulation of secondary metabolite biosynthesis in myxobacteria. These research efforts paved the way to sophisticated in vitro studies and to the heterologous expression of complete biosynthetic pathways in conjunction with their targeted manipulation. The progress made is a prerequisite for using the vast resource of myxobacterial diversity regarding secondary metabolism more efficiently in the future.

Keywords Myxobacteria · Secondary metabolism · Polyketides · Non-ribosomally made peptides · Heterologous expression · Biosynthesis gene cluster

Introduction

Not least owing to the pioneering work of the Höfle and Reichenbach research groups at the German Research Center for Biotechnology (GBF, Braunschweig, Germany), myxobacteria have become known as prolific producers of interesting and biologically active secondary metabolites. More than 7,500 different myxobacteria have been isolated and numerous strains have been analysed chemically [16]. From these, more than 100 new core structures plus approximately 500 derivatives have been described which has been discussed recently in several reviews [16, 57, 58]. The advanced exploitation of this resource of chemical diversity is of especial interest because the mode of action of natural products from myxobacteria is often unusual as they target cellular structures which are rarely or not at all hit by other secondary metabolites. Prominent examples are several compounds that interact with the eukaryotic cytoskeleton. Epothilone from *Sorangium cellulosum* is about to be approved for breast cancer treatment because it is a paclitaxel mimetic [24]. Furthermore, this compound can be used to treat paclitaxel-resistant tumors, shows good water solubility and can be produced by fermentation. Epothilone stabilizes microtubuli in the cell disabling the assembly of functional mitotic spindles required for cell proliferation and thus resulting in the induction of apoptosis [24, 57]. Since the isolation of epothilone additional myxobacterial compounds have been found that exhibit the opposite mode of action by destabilizing microtubuli (i.e. disorazol [9], tubulysin [64]) or interfere with the actin skeleton (i.e. rhizopodin [17], chondramid [63]).

Furthermore, secondary metabolites from myxobacteria often show structural elements which are rarely produced by other sources. Most of the isolated compounds represent hybrids of polyketides (PKs) and non-ribosomally made peptides (NRPs), whereas pure PKs are only rarely reported [70]. Examples for pure PKs are the aurafurons [40], tuscolid, tuscuron [46] and dawenol [73].

In contrast to several secondary metabolites from actinomycetes [60], myxobacterial natural products often lack glycosylations and other biosynthetic steps that take place after the assembly of the core structures. An exception to this general finding is the cytotoxic compound chivosazol bearing a 6-deoxyglucose moiety attached to the aglycon [29, 32, 51]. An example for the chemical diversity generated by myxobacterial secondary metabolism without using typical post-PKS/NRPS steps are the leupyrrins which are derived from a mixed PKs/peptide/isoprenoid biosynthesis. In this case several

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precursors are assembled, furthermore functionalized and rearranged providing an impressive example for natural combinatorial biosynthesis [5, 6].

The modern era of myxobacterial secondary metabolism research—impact of genomics and molecular biology

Due to their unusual life cycle, culminating in the formation of sometimes highly complex fruiting bodies and their gliding ability, myxobacteria have been studied extensively using microbiological and genetic techniques during the last 40 years. Antibiotic activity was described from myxobacteria as early as 1947 [49] and the first myxobacterial secondary metabolite ambruticin was isolated in 1977 [59]. However, the molecular era of myxobacterial secondary metabolism research did not start before 1995 when the biosynthetic gene cluster for the production of saframycin was identified [55]. Today, 17 natural product biosynthetic gene clusters from myxobacteria have been published, some of them were even reported twice (Fig. 1, Table 1) [7, 10, 35, 38, 44, 74, 77].

The high potential of myxobacteria as potent producers of secondary metabolites is exemplified by the most studied representative *Myxococcus xanthus* DK1622. This strain is the model organisms for studying the fruiting body formation and motility in myxobacteria. Despite the yellow appearance of *M. xanthus* DK1622, it was thought to be a non-producer of secondary metabolites based on investigations in several laboratories in the past 30 years. In contrast, a recent detailed analysis of the genome sequence revealed at least 18 different biosynthetic gene clusters for the production of secondary metabolites [4], most of them harbouring polyketide synthase (PKSs) genes and non-ribosomal peptide synthetase (NRPSs) genes as was previously also shown for other myxobacteria [70]. From these gene clusters, at least 14 are located in a 1.6 Mbp super-cluster (found between 3.2 and 5.8 Mb on the genome). However, no evidence for plasmid or chromosome fusion leading to the observed super-clustering has been found (http://www.tigr.org). Therefore, more than 8.5% of the genome is dedicated to secondary metabolism which is more than reported in the well-known secondary metabolite producers *Streptomyces coelicolor* [2] and *S. avermitilis* [27, 48] (4.5 and 6.6%, respectively).

Some of the identified biosynthetic genes show striking homology to known gene clusters from other myxobacteria and subsequently several of the corresponding compounds have been isolated and identified after large-scale cultivation (P. Meiser, HBB, RM, unpublished results). Besides the myxalamids [30, 71] as major compounds produced in liquid media, the iron siderophore myxochelin B [11, 39, 69], myxovirescins [75] and different myxochromides [82] have been isolated and characterized. Further compounds from DK1622 have been identified and their structures are currently being elucidated in our laboratory.

Similarly, numerous biosynthetic gene clusters of currently unknown function have been identified in the genomes of *S. cellulosum* So ce56 (which was sequenced by a German consortium; http://www.genetik.uni-bielefeld.de/GenoMik/cluster6.html) and *Stigmatella aurantiaca* DW4/3-1 (sequenced by TIGR; http://www.tigr.org). These strains are well known as producers of various secondary metabolites and some corresponding biosynthetic gene clusters have already been identified [15, 36, 40, 51, 68, 81].

Since the first molecular biological studies on myxobacterial secondary metabolism in *M. xanthus* [50, 55] and *S. aurantiaca* [3, 68], almost every related publication described highly unusual genetic and biochemical features [79]. Studies on the biosynthesis of stigmatellin in *S. aurantiaca* Sg a15 revealed the first example of an exclusively iterative acting module in a type I PKS (Fig. 2a) [12, 45], whereas studies dealing with the biosynthesis of myxochromides in *S. aurantiaca* DW4/3-1 and *M. xanthus* DK1622 provided evidence for the skipping of a complete NRPS module which had never been observed before (Fig. 2b) [81, 82]. A detailed understanding of the underlying mechanisms is the prerequisite to use these and other biochemical principles in a targeted manner in the future.

Besides its interesting biological activity as acetyl-CoA carboxylase inhibitor, soraphen from *S. cellulosum* is one of the few natural products, in general, that require benzoyl-CoA as a biosynthetic starting unit to generate a phenyl-substituted macrolide [1, 23]. Phenyl substituents are structural moieties preferentially used by medicinal chemist as starting points to modify natural products and their properties. Consequently, the biosynthetic genes for soraphen were identified [67] and the DNA fragment encoding the loading module of the soraphen PKS was fused with elongation modules from an actinomycete biosynthetic gene cluster resulting in the production of novel phenyl substituted products after supplementation of the newly constructed strain with benzoic acid [83]. In addition, the feeding of chemically modified benzoic acid derivatives led to the formation of the expected altered compounds in the same system [13] and in the original producer [22].

In vitro studies also play an important role in determining enzyme specificity and/or promiscuity leading to information which can be used to generate modified analogues of selected secondary metabolites. Exemplarily, the Walsh group [47] generated analogues of early intermediates in epothilone biosynthesis by incubation of various activated thioesters with the required recombinant enzymes (Fig. 3). However, it needs to be shown whether the observed in vitro promiscuity of the initial enzymes can be used to obtain complete modified epothilones in vivo.

In vitro experiments with purified enzymes have also been used to confirm or elucidate complete biochemical pathways involved in myxobacterial secondary metabolism. The iron siderophore myxochelin was synthesized in vitro after the production of activated MxcEFG in

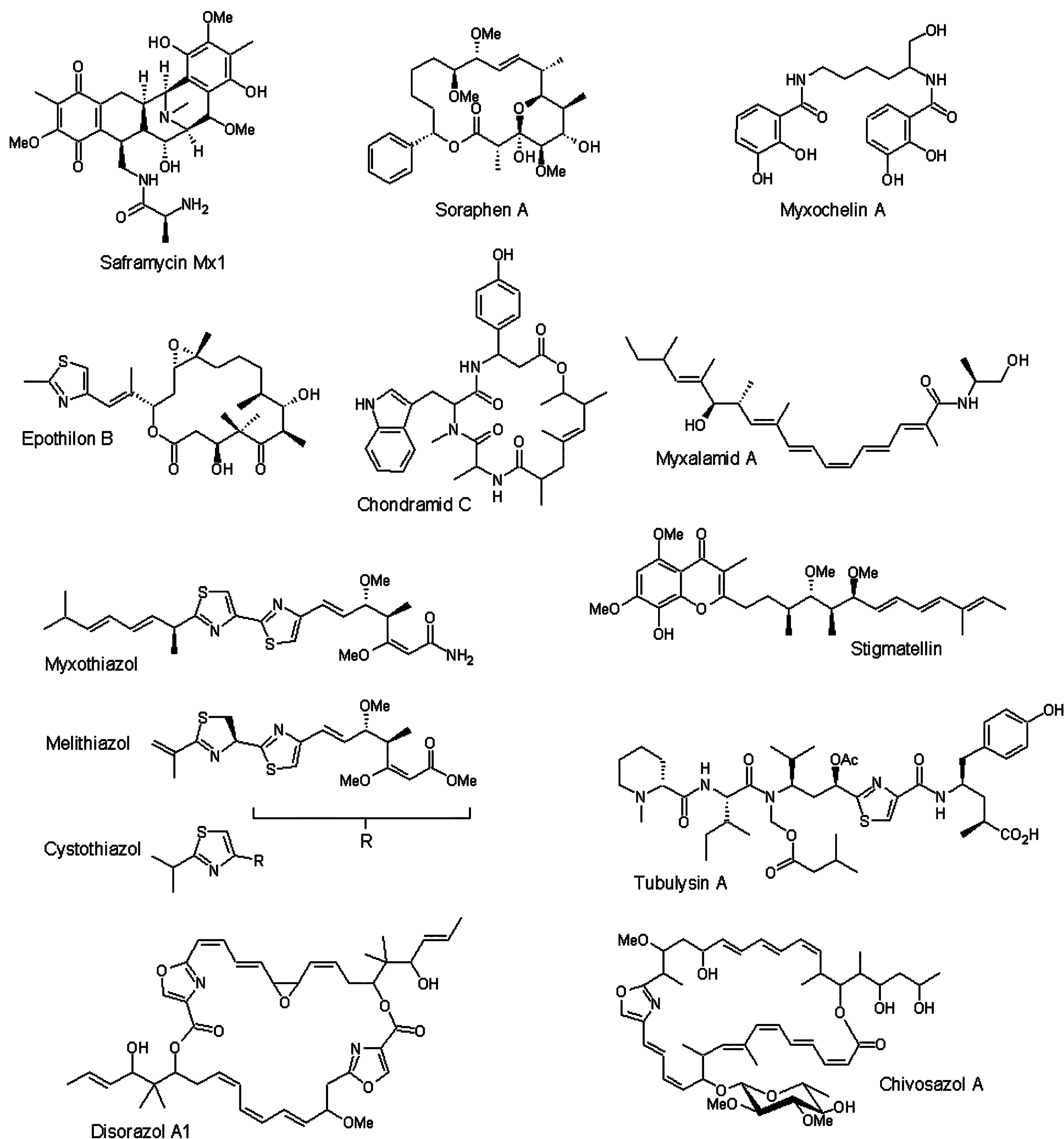

Fig. 1 Myxobacterial secondary metabolites whose biosynthetic gene clusters have been published. Structures of myxochromides A and S see Fig. 2b

Escherichia coli and subsequent incubation with the required substrates. These experiments provide direct evidence for the postulated biosynthetic mechanism [69] including an iterative use of a condensation domain and an unusual reductive chain termination mechanism (Fig. 4a) [11]. A similar release mechanism has been postulated for the myxalamids (Fig. 4b) and the saframycins [54, 71]. Additionally, another unprecedented chain termination mechanism also combined with methyl ester formation in the biosynthesis of myxothiazol and melithiazol has been identified in vivo using mutants deficient in the formation of the required enzymes and subsequently confirmed by in vitro experiments (I. Müller et al. submitted). The terminal amide

Table 1 Complete biosynthetic gene clusters identified from myxobacteria

Compound	Type	Producer	Year	Reference
Saframycin	NRPS	*Myxococcus xanthus* DSM504/15	1995/1996	[54, 55]
Soraphen	PKS	*Sorangium cellulosum* So ce26	1995/2002	[43, 67]
Myxothiazol	PKS/NRPS	*Stigmatella aurantiaca* DW4/3-1	1999	[68]
Myxochelin	NRPS	*Stigmatella aurantiaca* Sg a15	2000	[69]
Epothilone	PKS/NRPS	*Sorangium cellulosum* So ce90	2000	[35, 44]
Myxalamid	PKS/NRPS	*Stigmatella aurantiaca* Sg a15	2001	[71]
Stigmatellin	PKS	*Stigmatella aurantiaca* Sg a15	2002	[12]
Melithiazol	PKS/NRPS	*Melittangium lichenicol*a Me l46	2003	[77]
Tubulysin	PKS/NRPS	*Angiococcus disciformis* An d48	2004	[61]
Disorazol	PKS/NRPS	*Sorangium cellulosum* So ce12	2005	[7, 38]
Chivosazol	PKS/NRPS	*Sorangium cellulosum* So ce56	2005	[51]
Cystothiazol	PKS/NRPS	*Cystobacter fuscus* AJ-13278	2005	[10]
Myxochromide S	PKS/NRPS	*Stigmatella aurantiaca* DW4/3-1	2005	[81]
Myxochromide A	PKS/NRPS	*Myxococcus xanthus* DK1622	2006	[82]
Chondramide	PKS/NRPS	*Chondromyces crocatus* Cm c5	2006	S. Rachid et al. (submitted)

found in both structures is derived from NRPS catalyzed glycine extension and subsequent oxidation which results in the formation of an unstable intermediate releasing the amide upon degradation (Fig. 4c). In vivo and in vitro studies show that the methyl ester found in myxothioazol Z and melithiazol A is then made by the amidase MelJ which hydrolyses the corresponding amide to the free acid and subsequent methylation of the free acid by the *O*-methyltransferase MelK.

A new PKS chain-release mechanism was postulated to take place during stigmatellin biosynthesis. However, to date, this theory is based only on the feeding experiments and the presense of a terminal domain that shows no homologies to other proteins in the databases (Fig. 4d) [12].

Heterologous expression of complex biosynthetic gene clusters as a tool to access the diversity of myxobacterial secondary metabolites

Not only in myxobacteria, genetic accessibility (construction of mutants, DNA manipulations) is the major limiting factor for the identification and manipulation of various secondary metabolite biosynthetic gene clusters [20]. Myxobacteria often grow slowly with doubling times between 16 and 24 h; they are naturally multiresistant against most commonly used antibiotics and often genetic methods established for one strain cannot be applied to others, even if they are phylogenetically closely related [37]. As plasmids have never been described from any myxobacterium, developing a toolkit of useful methods and vectors for working in the native host turned out to be problematic. Taken together, these disadvantages and the fact that gene clusters for secondary metabolites can easily reach sizes of more than 100 kbp, manipulation of the natural producer and heterologous expression are challenging tasks. However, with myxobacterial strain collections available and the given potency of the produced compounds, there is an urgent need to access the genomic potential of these bacteria on the molecular level which is why a generalized approach to gene cluster cloning and modification would be highly desirable.

A promising way to reach this goal would be to establish an efficient method of cloning the desired gene clusters and subsequent heterologous expression in a suitable host. The epothilone gene cluster was identified and analysed by scientists at Novartis [44] and KOSAN biosciences in parallel. The latter group cloned the respective genes onto two plasmids and expressed them in *S. coelicolor* [74]. The construction of the expression plasmids included several rounds of cloning and the resulting strain produced epothilone only in trace amounts (50 µg/l compared to 20 mg/l in the original natural producer). Nevertheless, this work showed that the expression of myxobacterial gene clusters in unrelated host bacteria is possible in general. Similar results were obtained for the production of soraphen A from *S. cellulosum* So ce26 in *S. lividans* ZX7 [86]. Here, a productivity of one-tenth of the amount made by the natural producer (3 mg/l) could be achieved but the cloning procedures were even more laborious and time consuming.

In order to obtain better production titers of epothilone, the KOSAN group used *M. xanthus* as the expression system and stepwise integrated the whole cluster for epothilone biosynthesis into the chromosome [34]. The initial production titers (0.3 mg/l) were much better than those in *S. coelicolor* and could be improved to the level of the original natural producer by optimization of the fermentation conditions [41]. However, it is noteworthy that in the meantime the titer in the natural producer has been increased dramatically by classical mutagenesis and media optimization to several hundred milligrams per litre (K. Gerth, unpublished result), as it has also been described for the production of soraphen in S. *cellulosum* (from 0.3 ml/l to 1.5 g/l) [16]. When the *epoK* gene encoding the cytochrome P450-dependent enzyme responsible for epoxidation of the epothilones C and D to the A and B forms, respectively, were mutated, the recombinant *M. xanthus* strain produced only

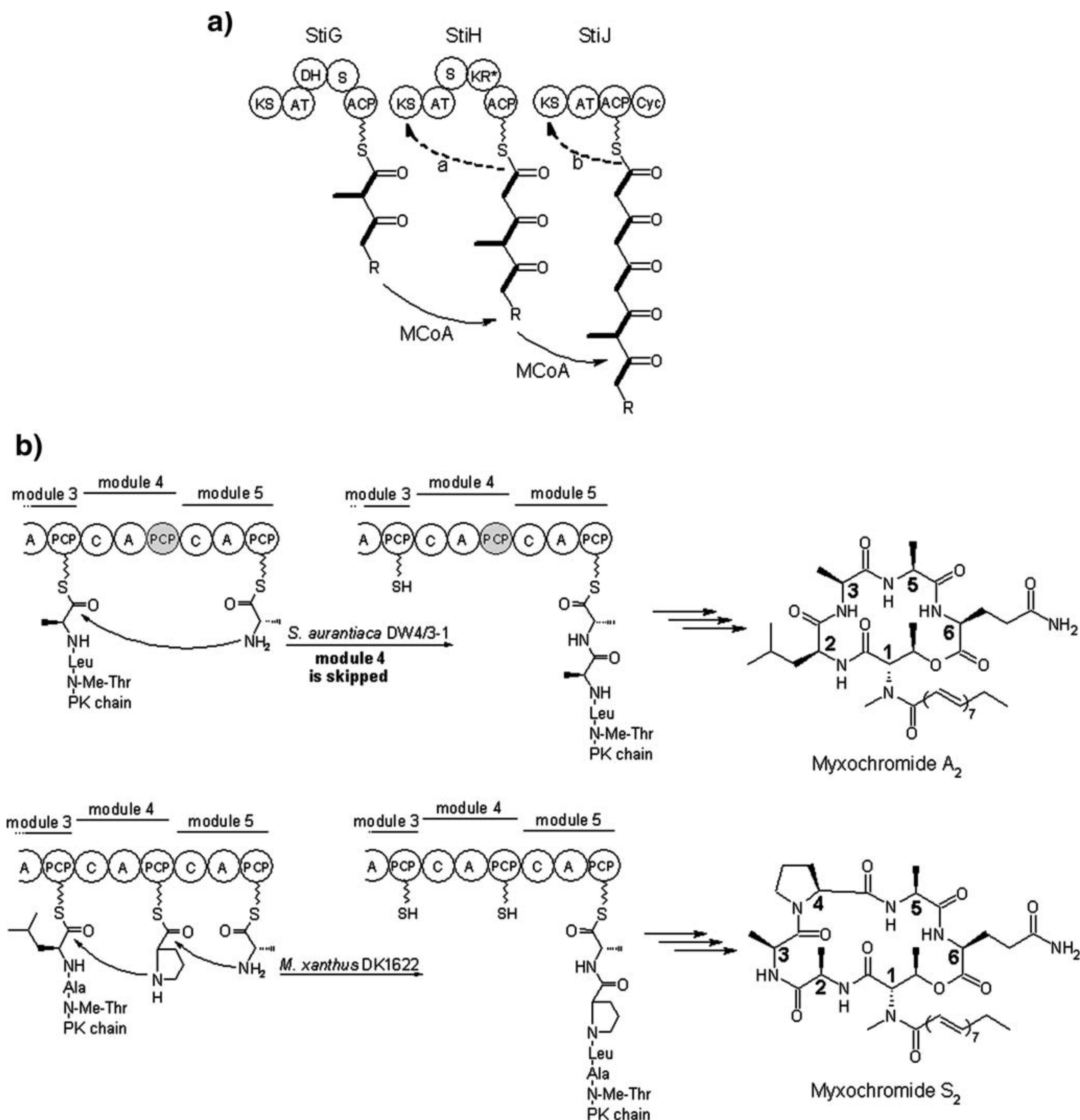

Fig. 2 Programmed ''stuttering'' and ''module skipping'' in myxobacteria. **a** The last enzyme bound intermediates in the stigmatellin biosynthesis are shown. *Dashed arrows* indicate either iterative use of StiH (**a**) or StiJ (**b**). MCoA (Malonyl-CoA), domains for acyltransferase (AT), acyl carrier protein (ACP), ketosynthase (KS), ketoreductase (KR), dehydratase (DH), spacer (S), cyclization (cyc), for R see Fig. 1, *asterisk* indicates an inactive domain. **b** The differences in the biosynthesis of myxochromide A and S are shown. The inactive PCP domain of module 4 in the myxochromide S biosynthesis is highlighted in grey [82]. The numbering in the structures corresponds to the modules incorporating the shown amino acids. PK (polyketide chain), domains for adenylation (A), peptidyl carrier protein (PCP), condensation (C)

epothilones C and D, with the more bioactive epothilone D as the major product [34].

The advantages of *M. xanthus* as heterologous host for the expression of other myxobacterial biosynthetic gene clusters are quite obvious: The codon usage and the physiology are very similar, gene cluster-specific promotors are likely to be active in *M. xanthus* and post-translational activation of the PKS and/or NRPS modules should be very efficient [78]. However, no plasmids are available and all required genes have to be

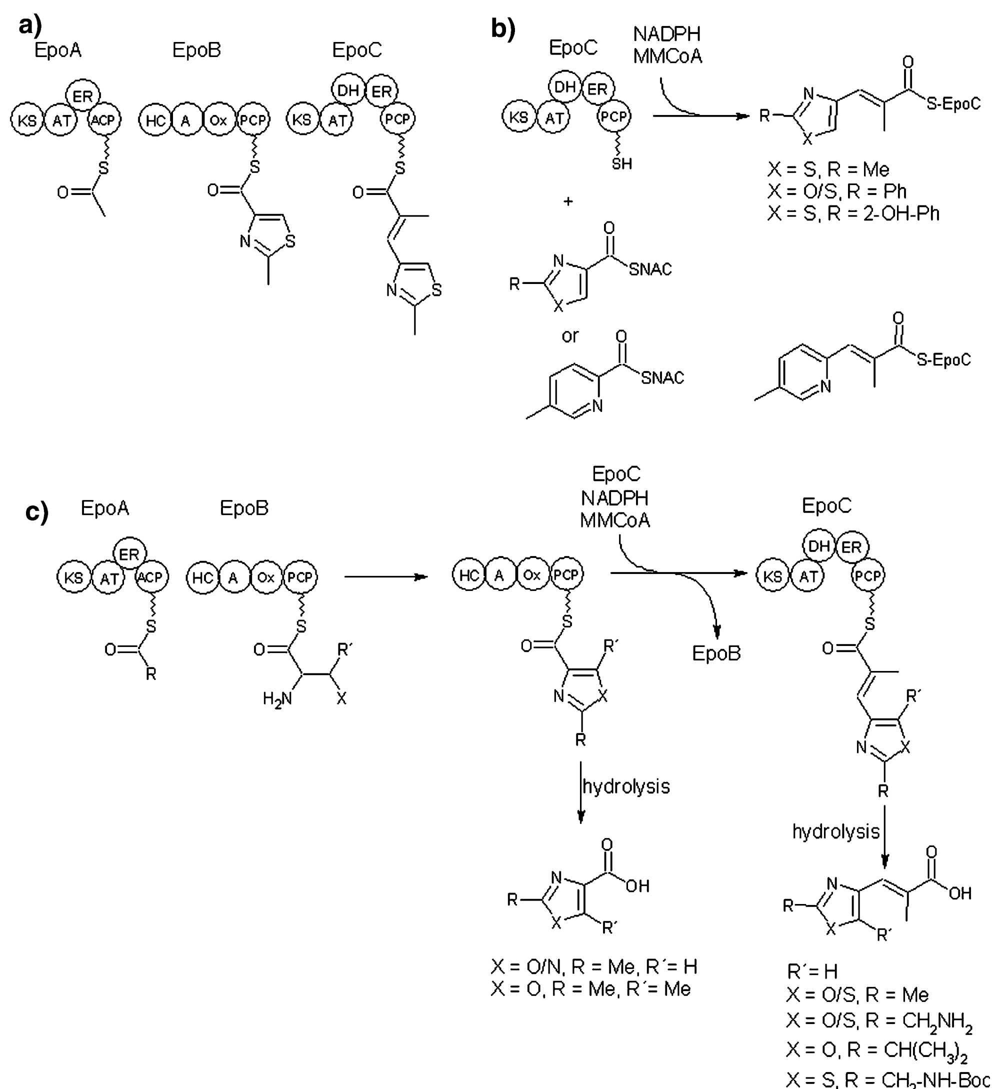

Fig. 3 Biosynthesis of epothilone intermediates with alternate starter units. **a** Natural start of epothilone biosynthesis [44]. **b** Generation of alternative starting units using EpoC and different *N*-acetylcysteamine thioesters (SNAC) of heterocyclic carboxylic acids [21]. **c** Similar work using EpoA, EpoB and EpoC (optional). In this case the products were detected after hydrolysis [66]. Methylmalonyl-CoA (MMCoA), *t*Butyloxycarbonyl (Boc); domains for enoylreductase (ER), heterocyclization (HC) and oxidation (Ox), other abbreviations see Fig. 2

integrated into the chromosome. Additionally, the doubling time of 4–5 h is still slow compared to other bacteria and *M. xanthus* has a strong tendency to produce high levels of ammonium during growth making fermentations difficult to perform [16]. A systematic search for fast-growing myxobacteria led to the isolation of several thermophilic strains belonging to almost all known myxobacterial species which are characterized by doubling times of less than 2 h [14].

Pseudomonads have recently been shown to be attractive alternative heterologous host organisms [80]. They can grow almost as fast as *E. coli*, show a very similar codon usage to myxobacteria and plasmids harbouring inducible promoters are available. Furthermore, it was shown that ‘‘foreign’’ PKSs and NRPSs are efficiently post-translationally activated by the pseudomonads intrinsic phosphopantetheinyl transferases [18]. The remaining obstacles in using pseudomonads as expression hosts are mostly related to the required exchange of regulatory elements and the enormous size of the biosynthetic gene clusters, which would obviously hamper their efficient cloning and modification. Both problems have been solved recently using Red/ET recombineering [84, 85] that allows the reassembly (‘‘stitching’’) of complete gene clusters from different cosmids plus the exchange of the promotor(s) as shown for myxochromide S production in *Pseudomonas putida* (Fig. 5) [80]. While the natural production in *S. aurantiaca* is only 8 mg/l after 7 days of fermentation, more than 40 mg/l have been obtained from *P. putida* after 2 days of growth. Additionally, this comparably high titer resulted in the production of previously unknown derivatives. Furthermore, the heterologous expression of this biosynthetic gene cluster unambiguously showed that the PKS involved is acting iteratively and that NRPS module 4 is most likely skipped during the biosynthesis, a process that has not been described previously (Fig. 2b).

The pseudomonads expression approach has also been used to activate ‘‘silent’’ biosynthetic genes from myxobacteria for the production of new natural products. In a proof of principle study, a type III PKS of

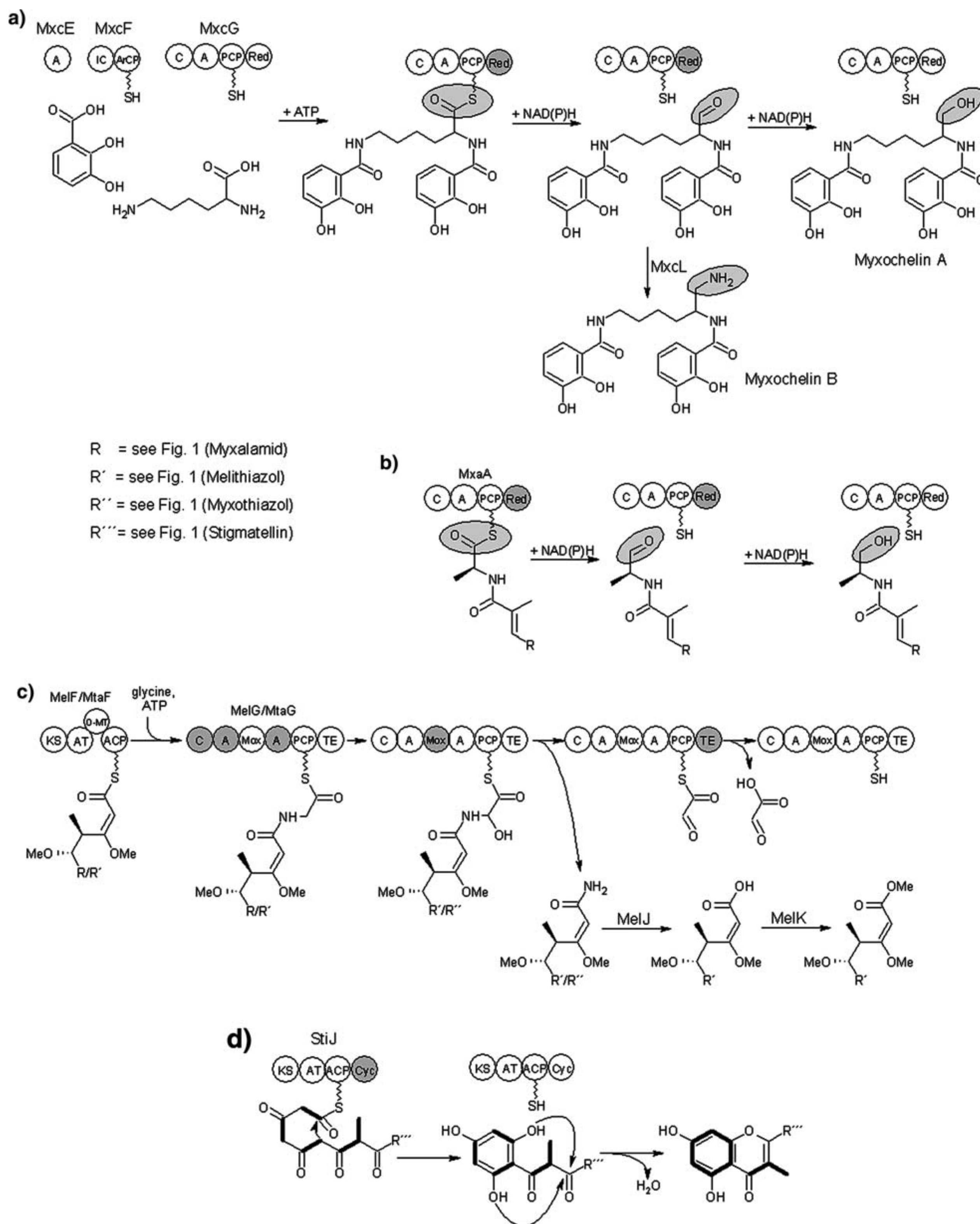

Fig. 4 Unusual chain termination mechanisms found in myxobacteria. Active domains are shown in grey. **a** The biosynthesis of myxochelin as confirmed by in vitro studies [11]. **b** A similar mechanism was postulated for the biosynthesis of the myxalamids [71]. The reduction of the thioester to the aldehyde might also take place in a two-step mechanism with an enzyme-bound *O*,*S*-hemiacetal as intermediate. **c** Postulated final steps in the biosynthesis of myxothiazol [68] and melithiazol [77] are in agreement with results from recent in vitro studies (I. Muller et al., submitted). **d** Postulated chromone ring formation in stigmatellin biosynthesis. Acetate units as determined from labelling experiments are shown in *bold* [12]. Domains for isochorismate synthase (IC), aryl carrier protein (ArCP), reduction (Red), monooxygenase (Mox), thioesterase (TE), other abbreviations see Fig. 2

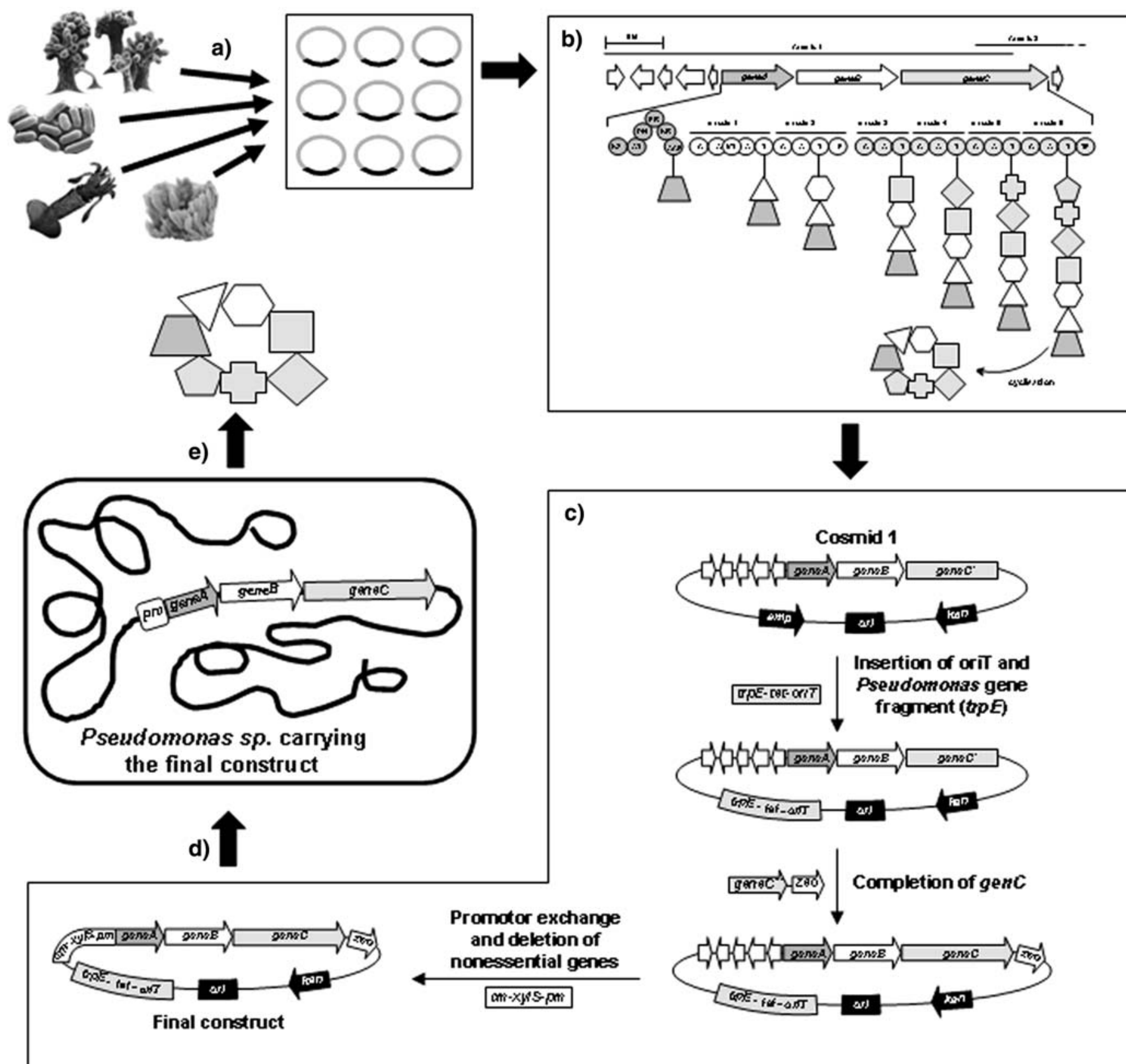

Fig. 5 General outline of the heterologous expression of biosynthetic gene clusters from non-accessible or difficult sources in pseudomonads as expression hosts: **a** The construction of gene libraries from various sources including metagenomic approaches; **b** Identification and analysis of the gene cluster; **c** Cloning of the complete cluster on one construct including promotor exchange for heterologous expression; **d** Transformation of the final construct into pseudomonads and **e** compound production in the heterologous host. Methyltransferase domain (MT), anthranilate synthase gene (*trpE*; non-essential in rich medium and conserved gene from *P. putida* used for integration into the chromosome), tetracycline/zeozin/chloramphenicol resistance genes (*tet*/*zeo*/*cm*), toluic acid inducible promotor (*pm*) with its regulator (*xylS*), other abbreviations see Fig. 2

unknown function from *S. cellulosum* So ce56 was expressed in *P. putida* and flaviolin could be isolated from these cultures in good yield (>6 mg/l) [19]. Although this compound is known from fungi and streptomycetes, it has never been identified from any myxobacterium. Additionally, a search for flaviolin or its precursors in extracts of *S. cellulosum* So ce56 grown under a variety of conditions did not result in the detection of the compound. These results clearly show that the heterologous expression approach of genes of unknown function is valuable for the production of novel natural products.

The powerful combination of advanced cloning techniques and an advantageous expression host (all of which are currently far from being optimized) will allow the production of several interesting myxobacterial secondary metabolites from the available strains in the future.

Four myxobacterial compounds (apicularen [33], saframycin [28], chondramide [31], rhizopodin [62]) with striking similarity to secondary metabolites isolated from higher marine organisms (i.e. sponges, molluscs) are known [4]. These findings raise the question of the origin of these marine compounds and in fact there is increasing evidence that marine myxobacteria exist [25, 26, 42] which might be responsible for the formation of these products. It is clear today that some other compounds isolated from marine higher organisms are indeed produced by bacterial symbionts living in close association with the eukaryote [53, 65]. The methods described above might allow the production of new compounds directly from environmental DNA-samples omitting the time consuming or sometimes impossible isolation of pure cultures of any kind of putative producer of natural products.

Regulation of myxobacterial secondary metabolism and outlook

Above, some examples of secondary metabolites from myxobacteria and the elucidated underlying principles of their biosynthesis have been presented. While some knowledge has been gained regarding this area of research, almost nothing is known about the regulation of secondary metabolite production in myxobacteria. In streptomycetes and fungi, secondary metabolites with antibiotic activity are often produced at the onset of the stationary growth phase and are therefore regarded as a defence mechanism to allow the progression of the life cycle and the protection of the produced spores [8]. This assumption seems to hold true for some myxobacterial compounds as well. Because of their unusual life style as organisms preying on bacteria and fungi, at least some of the known secondary metabolites from myxobacteria might be involved in killing prey which is then made accessible as nutrient source using exoenzymes released by the cells [52]. The function of compounds that are not antibiotics is a point of interesting debate with many interesting theories.

The produced amounts of some secondary metabolites might be too low to detect under vegetative or developmental conditions and therefore the long-term goal to understand the regulation of myxobacterial secondary metabolite formation will help to increase or induce natural product formation. Initially, the effect of the composition of growth media needs to be carefully monitored as has been performed in a recent study on *S. cellulosum* So ce56 [15]. In a parallel study, it was shown by quantitative RT-PCR that the chivosazol biosynthetic gene cluster in *S. cellulosum* So ce56 is differently regulated depending on the growth medium applied [36]. The corresponding transcript was shown to be very stable and transcription could be measured from the onset of each experiment, albeit with significant induction at different time points.

Another example of a study dealing with the regulation of secondary metabolite formation is the identification of a positive regulator of stigmatellin biosynthesis in *Cystobacter fuscus*. After transposon mutagenesis 1,200 mutants have been screened using bioassays enabling the detection of decrease or loss of either argyrin or stigmatellin biosynthesis. Several mutants have been identified which show a strongly reduced or even completely abolished stigmatellin production. In addition to the identification of the stigmatellin biosynthetic gene cluster, the novel positive regulator StiR related to sigmatellin production was identified after transposon recovery from the chromosome of a mutant [56]. StiR shows some similarity to two-component sensor histidine kinases and its role in regulating the stigmatellin biosynthesis was confirmed by reconstruction of a genotypically identical mutant by double crossover experiments. However, the regulatory cascade involving StiR has not been elucidated in detail. In other bacteria several two-component regulatory systems have been described as involved in the secondary metabolite production [72, 76] and interestingly the number of this type of genes is high in *M. xanthus* and *S. cellulosum*. Functional genomics has only just started with the recently finished genome sequences of these two myxobacterial strains [plus *S. aurantiaca* DW4/3-1, which has been sequenced to a fourfold coverage (http://www.tigr.org)]. In the future, this information in combination with the possibility to clone the whole biosynthetic gene clusters into optimized expression hosts will strongly speed-up myxobacterial natural product research.

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