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Review

Genetic manipulation and tools in myxobacteria for the exploitation of secondary metabolism

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ABSTRACT

Myxobacteria are famous for their capacity for social behavior and natural product biosynthesis. The unique sociality of myxobacteria is not only an intriguing scientific topic but also the main limiting factor for their manipulation. After more than half a century of research, a series of genetic techniques for myxobacteria have been developed, rendering these mysterious bacteria manipulable. Here, we review the advances in genetic manipulation of myxobacteria, with a particular focus on the exploitation of secondary metabolism. We emphasize the necessity and urgency of constructing the myxobacterial chassis for synthetic biology research and the exploitation of untapped secondary metabolism.

1. Introduction

Although previously misidentified as fungi in the early 20th century, myxobacteria, a type of bacteria, have been regarded as mysterious organisms due to their social behaviors. These include swarm-gliding movements, morphogenesis of multicellular fruiting body structures, and wolf-like predation, which are rare in bacteria but common in eukaryotes. Myxobacteria are thus used as amenable model organisms in the study of prokaryotic multicellularity and cell–cell communication to investigate the involved molecular mechanisms and their significance in evolution and ecology [1–4]. In addition, myxobacteria are a prolific source of novel natural compounds with great potential for discovering new drugs with chemical diversity and uncommon mechanisms of action [5–7]. Myxobacteria typically have a large genome with numerous genes devoted to the biosynthesis of secondary metabolites; however, most of these genes have yet to be identified [8,9]. They are a group of predatory microorganisms that are ecologically applicable in biocontrol; for instance, a *Corallococcus* sp. was recently shown to attack pathogenic fungi by lysing cells with β-1,6-glucanase (GluM), as well as to regulate soil microbial community and control cucumber fusarium wilt [3,10]. *Myxococcus xanthus* DK1622 has also been shown to inhibit the growth of certain plant-pathogenic fungi using a toxin-immune protein system [11]. Thus, myxobacteria are relevant to both fundamental and applied researches. However, the unique sociality of myxobacteria is not only an intriguing scientific topic but also a limiting factor for their manipulation.

Myxobacteria typically grow slowly and rarely form colonies from a single cell [1]. It is, therefore, difficult to isolate myxobacterial strains, although they are among the most abundant and ubiquitous bacteria (accounting for 2% of the total OTUs) in soils worldwide [12]. Myxobacteria are one of the least cultured bacteria, and many unclassified myxobacteria exist [13]. The lack of genetic tools severely hampered research on cultured myxobacteria, particularly for non-model strains. Compared to common model strains such as *Escherichia coli*, myxobacteria lack efficient genetic manipulation techniques and selection markers, making it difficult to construct, screen, and purify mutant strains. Nevertheless, after more than half a century of research, several genetic tools have been developed and optimized in myxobacteria, primarily in the model species *Myxococcus xanthus*. These effective technologies facilitate myxobacteria research and exploitation. In this review, we provide a brief description of myxobacteria and their secondary metabolism and summarize the genetic tools developed in myxobacteria and their applications in the study of natural products, particularly epothilones. We propose myxobacterial chassis for synthetic biology research and genetic tools in non-model myxobacteria for untapped secondary metabolism.

2. Social and developmental biology of the myxobacteria

Myxobacteria are a group of gliding gram-negative bacteria phylogenetically related to the *Delta-proteobacteria* order Myxococcales. *Myxococcales* contains three validly published suborders, *Cystobacterineae, Sorangiineae*, and *Nannocystineae*, which are subdivided into 10 families, 29 genera, and 58 species [14]. Recently, the *Myxococcales* was reclassified as the *Myxococcota* phylum based on 120 conserved single-copy marker genes and rRNA genes [15].

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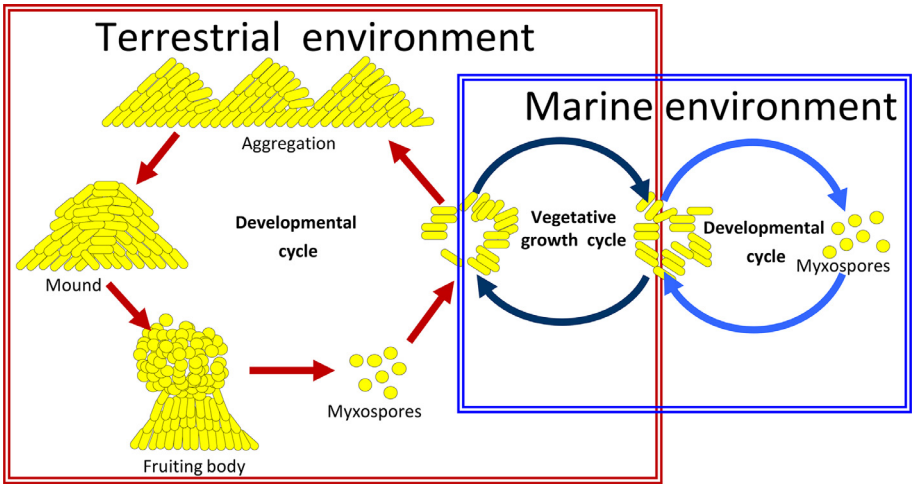

Fig. 1. A schematic diagram showing the life pattern shift of halotolerant *Myxococcus fulvus* HW-1 between the marine and terrestrial conditions. The marine environment is saline, like seawater, whereas the terrestrial environment is relatively low in salt. In both cases, *M. fulvus* HW-1 grows (vegetative growth cycle) under eutrophic conditions and develops into fruiting bodies or myxospores (developmental cycle) with exhausted nutrition.

Myxobacteria secrete enzymes capable of degrading various biological macromolecules or living microbial cells [16]. Myxobacteria are traditionally divided into two groups based on their feeding habits: bacteriolytic myxobacteria, which degrade living microorganisms, and cellulolytic myxobacteria, which decompose cellulose and dead cells. The feeding pattern is still the basis for myxobacteria isolation techniques [17]. To date, most cultured myxobacteria are bacteriolytic, and only two genera, *Sorangium* and *Byssovorax* [14,18], comprise the cellulolytic myxobacteria. Notably, some recently identified myxobacteria, including *Vulgatibacter incomptus, Labilithrix luteola*, and *Simulacricoccus ruber*, neither utilize living bacterial cells nor hydrolyze cellulose [19,20].

The motility of myxobacteria is driven by two distinct mechanisms: social motility (S motility), cells moving in large clusters mediated by the extending and retracting of polar type IV pili, and adventurous motility (A motility), cells moving as individuals driven by laterally embedded motility engines [2,21]. The A- and S-motilities collaborate to permit frequent encounters with prey, thereby facilitating efficient predation [16,22]. Under conditions of starvation, most myxobacteria can develop fruiting body structures: the developing cells migrate into aggregation centers and climb on top of each other to form fruiting bodies, where parts of cells inside cysts differentiate into environmentally resistant myxospores [23]. Notably, while myxospores of the model strain *Myxococcus xanthus* DK1622 can germinate from single cells when cultured in appropriate media, the germination of many other myxobacterial strains, particularly new isolates, is normally cell density-dependent even in these 'appropriate' media, and the fruiting body structures may ensure sufficient cell numbers for germination [17]. Notably, some recently discovered myxobacteria, such as *Anaeromyxobacter* [24], *V. incomptus, L. luteola* [19], *S. ruber* [20], and *Polyangium aurulentum* [25], are incapable of fruiting, and the non-fruiting myxobacteria are probably widespread in the environment, representing most of the uncultured myxobacteria in nature [26].

Although appealing, the multicellular social characteristics of myxobacteria are the primary factors limiting their manipulation. Li et al. reported a simple method for isolating salt-tolerant myxobacteria from marine samples. These salt-tolerant myxobacteria could grow but could not produce fruiting bodies at high salt concentrations [27]. Further research revealed that salt-tolerant *Myxococcus fulvus* HW-1 rapidly adapted to different living environments: cells grew normally and formed complete fruiting body structures in terrestrial environments (with low concentrations of seawater or salts); cells became shorter and formed rudimentary structures or even simple cell mounds as the seawater concentration increased [28–32]. Fig. 1 depicts the living pattern shift of HW-1 in response to salinity. The observed changes in fruiting body formation ability and cell density-dependent growth of salt-tolerant *Myxococcus* strains are indicative of ecological adaptation strategies of myxobacterial social behaviors to marine environments. Moreover, myxobacteria could be isolated and genetically modified by utilizing their simple living pattern in specific environments.

3. Diversity of secondary metabolism in myxobacteria

Microorganisms, particularly medicinal resource microorganisms rich in biosynthetic pathways of secondary metabolites, are consistently among the most promising sources for developing new drugs or lead compounds. Myxobacteria are a relatively new group of medicinal microorganisms with complex structures and diverse action mechanisms [33], whereas actinobacteria have been extensively screened for antibiotics for more than half a century. 109 distinct core structures and more than 600 derivatives have been determined from the normal screening of approximately 7500 identified myxobacterial strains; the genus *Sorangium* produces the most abundant secondary metabolites, accounting for 48.4% of the total products found in myxobacteria, followed by *Myxococcus* (20.7%), *Chondromycetes* (10.3%), *Stigmatella* (6.9%) and *Polyangium* (5.2%) [6].

Myxobacterial bioactive compounds have various chemical structures, but most are the derivatives of products synthesized by polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), or hybrid PKS–NRPS systems [34]. The stepwise synthesis of polyketides, non-ribosomal peptides, and their hybrids involves the assembly of acyl-CoA thioester (in the case of polyketides) or amino acids (proteinogenic and non-proteinogenic in the case of non-ribosomal peptides). Diverse modifications may occur during assembly or after the release of a compound, resulting in products with complex chemical structures. Thus, a fundamental core structure can generate numerous analogs. More than 70 naturally occurring structural variants of soraphen were once discovered in *S. cellulosum* So ce26 [35]. Myxobacterial metabolites are also strain-specific; in *S. cellulosum* So ce90/B2, 37 analogs of epothilone have been isolated [36,37]. Li and colleagues identified 23 epothilone analogs from strain So0157-2 [38–42]. Only epothilones A, B, and C are present in So ce90/B2, whereas the others are unique (Figs. 2 and 3).

Moreover, myxobacterial products frequently exhibit diverse and novel action mechanisms, making them promising as new drugs for various applications, including antifungal, antibacterial, cytotoxic, and antiviral activities [43]. For example, bacterial RNA polymerase inhibitors such as thiolutin [44], streptolydigin [45], and holomycin [46] inhibit RNA synthesis in a manner distinct from that of the clinical drug rifamycin. Since they have no cross-resistance with rifamycin, these compounds have the potential to combat rifamycin resistance [47]. Epothilones, a type of microtubule stabilizer that mimics paclitaxel [36], exhibit potent anti-paclitaxel-resistant tumor activity [48]. Two epothilone varieties, Ixabepilone [49] and Utidelone [50] have

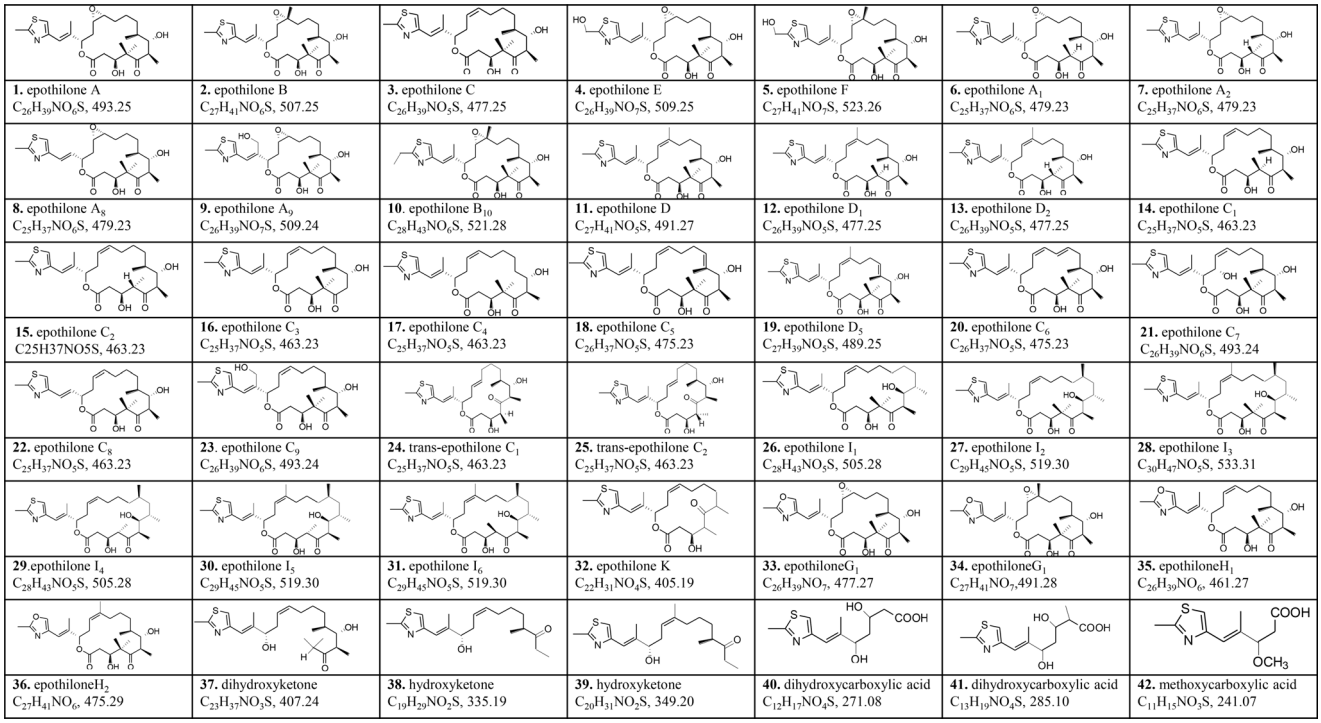

Fig. 2. Epothilone analogs identified in *S. cellulosum* So0157-2.

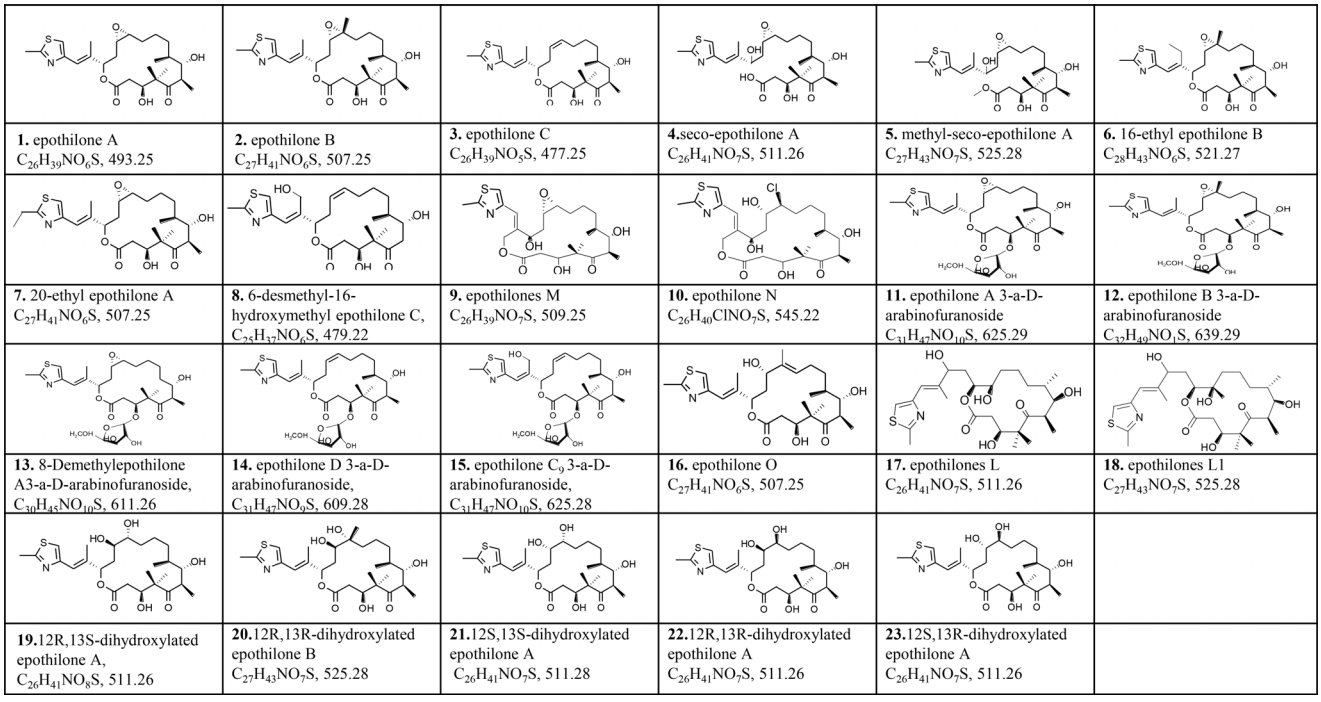

Fig. 3. Epothilone analogs revealed in *S. cellulosum* So ce90/B2.

been approved for use as antitumor drugs against advanced breast cancer in clinical settings. Epothilones are also promising in treating neurodegenerative disorders [51,52]. Several excellent reviews summarize the bioactivities and mechanisms of action of myxobacterial secondary metabolites, such as inhibitions of respiration, protein or nucleic acid synthesis, interferences of the protein phosphorylation system, electron transport, cell division, lipid synthesis, and carbohydrate metabolism [6,33,43,53].

Myxobacterial metabolites are highly specific. Althiomycin [54], saframycin [55], and pyrrolnitrin [56] are the only chemical core structures from myxobacteria that have been identified in other microbial resources. Similarly, approximately 40% of myxobacteria-derived compounds represent novel chemical structures that act on novel targets distinct from those imposed by compounds derived from *Actinomycetes, Bacillus*, or *Pseudomonas* [43]. The specificity of myxobacterial metabolites represents a significant opportunity for the discovery of new drugs.

A single myxobacteria species or strain is capable of producing numerous active secondary metabolites with distinct structural scaffolds. *S. cellulosum* strains have been found to contain a wide variety of compound structures, including macrolides (such as disorazol, chivosazol, soraphen, and epothilone), aromatics (such as jerangolid), quinones (such as sorangiolid), and polyenes (such as ratjadon and ambruticin) [6]. At least nine types of active natural products have been identified in *Chondromyces crocatus* Cm c5, including ajudazols, chondrochlorens, dialkylpyrazines, and thuggacins [7]. In the model strain *M. xanthus* DK1622, nine compounds with different types of structure have been found, of which Dkxanthenes, myxoprincomides, and homospermidin lipids are presently unique in DK1622, while myxothiazol, an inhibitor of mitochondrial respiration, has also been identified in *Stigmatella aurantiaca* [57], *M. fulvus* [58], and *Angiococcus disciformis* [59], and myxolamids are similarly produced in *M. fulvus, M. xanthus, S. aurantiaca*, and *C. coralloides* [34]. Systematic characterization of a single myxobacterial species or strain requires dogged efforts using the strategy of "one strain many compounds" (OSMAC) [60]. Examining strains from new genera rather than additional representatives within the same genus [61] increases the likelihood of discovering novel metabolites, but screening myxobacterial isolates from different environments could also reveal new active natural products such as nicotinic myxochelins [62], myxadazoles [63], and archangiumide [64].

With the advent of the genomic era, studies on the genome and the exploitation of secondary metabolite biosynthetic gene clusters (smBGCs) with undefined functions have gained attention as a novel strategy for expanding the range and diversity of bacterial secondary metabolites. According to the published genome information in the NCBI database, 91 cultured myxobacterial strains have been sequenced thus far. Except for a few small genomes of approximately 5 Mb [19,25,65], myxobacterial genomes range between 9 and 16 Mb in size. For example, the genomes of *Minicystis rosea* DSM 24000 [66] and *S. cellulosum* So0157-2 [67] are 16.04 Mb and 14.78 Mb, respectively, making them the two largest known prokaryotic genomes, surpassing many eukaryotic genomes such as *Saccharomyces cerevisiae* (12.16 Mb). A total of 42 myxobacteria have been fully sequenced, and their smBGCs have been analyzed using antiSMASH. As shown in Table 1, myxobacterial genomes contain more than 20 smBGCs, with the exception of the smallest genomes. In a specific strain of myxobacteria, such as the best-characterized *M. xanthus* DK1622, only nine of the 24 smBGCs and their products have been identified. Myxobacteria still contain a large number of metabolites awaiting further exploitation. Furthermore, considering the large number of uncultured myxobacteria and their unidentified secondary metabolic genes, the bioactive compounds reported to date from myxobacteria are merely the tip of the iceberg. Although omics-based research will reveal ever-increasing synthetic potentials of metabolites in myxobacteria [68], a thorough exploration of the potentials of drugs or leading compounds requires the development of efficient genetic tools for myxobacteria.

4. Genetic tools in myxobacteria

Exploitation of myxobacteria lags far behind that of other medicinal microorganisms, such as *Actinomycetes* and *Bacillus*, due to their slow and aggregate growth, complex multicellular behavior, and especially lack of efficient genetic methods. In conjunction with the genetic and biological research conducted on myxobacteria for more than half a century, several genetic manipulation techniques and tools have been developed, particularly for the model organism *M. xanthus*. The advantages and disadvantages of each of these tools are summarized in Table 2.

4.1. Gene delivery systems in myxobacteria

In bacteria, DNA is transported primarily through three mechanisms: transduction, transformation, and conjugation [69], and myxobacteria have demonstrated maneuverability with these gene delivery methods. The use of phage-dependent transduction for genetic modification of myxobacteria was once widespread but has since fallen out of favor due to its cumbersome nature and the advent of more effective techniques. Conjugation is predominantly utilized in *S. cellulosum*, but the operation method is strain-specific and lacks commonality. Transformation (electroporation rather than heat-shock transformation) stands out for its high efficiency in transporting DNA, particularly large fragments, and has become the primary method of gene delivery in myxobacteria.

4.1.1. Transduction

Transduction refers to the phage-mediated transfer of DNA from a donor cell to a recipient. *E. coli* phage P1 was the first phage to successfully introduce foreign DNA into an *M. xanthus* strain [70]. It allows the transfer of the transposon Tn5, which integrates randomly into the recipient chromosome to produce mutations [71,72]. In 1966, Burchard and Dworkin reported the first myxophage Mx1 from *M. xanthus* [73]. More than 35 myxophages have been isolated and classified into five serological groups: Mx1, Mx4, Mx8, Mx9, and Mxα [74]. Several generalized transduction phages have been derived [75–77], and Mx8 and Mx4 are capable of packaging up to approximately 50 kb of DNA [75]. Most of these studies on myxophages were conducted between 30–50 years ago, and myxophage-based transduction has been gradually phased out as more effective techniques have been developed. However, myxophage-derived integrases are still commonly used in myxobacteria gene editing, as will be discussed in detail in the "Site-specific recombination" section.

4.1.2. Transformation

Natural transformation is a significant way for bacteria to acquire genetic material in nature and a significant evolutionary force [78]. Shuttle plasmid pZJY41, which is derived from the autonomously replicating plasmid pMF1 of *M. fulvus* 124B02 [79], resulted in a successful transformation from *E. coli* to *M. xanthus* [80], according to a study conducted on *M. xanthus*. Exopolysaccharide (EPS), as an extracellular barrier, and restriction-modification system, as an inner barrier, both prevent the uptake of foreign DNA fragments, resulting in low efficiency of natural transformation, according to the authors.

Compared to natural transformation, electroporation employs transient high-voltage electrical pulses to induce the temporary formation of water channels or membrane pores on the cell membrane, allowing extracellular DNA to enter the cell. Electroporation has been developed as a fast, simple, and efficient technique for bacteria, yeast, plant cells, and mammalian cell lines [81]. Electrotransformation was first used in 1989 to introduce a plasmid containing 14 kb of *M. xanthus* DK101 genome DNA into development-deficient *M. xanthus* cells, producing fruiting transformants [82]. Subsequently, electrotransformation was optimized and widely adopted, eventually becoming the most prevalent method for transferring foreign DNA into *M. xanthus* cells [83]. For illustrative purposes, we highlighted several recent studies. With electroporation, the plasmids carrying target DNA of various sizes are efficiently transferred into myxobacteria, including smBGCs spanning tens or even a hundred kb [84]. In order to heterologously express myxobacterial secondary metabolites, the entire smBGC is assembled in *E. coli* cells using Red/ET recombineering technology or derivative technologies [87]. The smBGC was cloned based on the recombination of genomic library plasmids or even directly captured, modified precisely in *E. coli*, and then electroporated into *M. xanthus* or other hosts for heterologous expression. Combining advanced DNA recombineering techniques with an efficient electroporation technique allows for the exploration of secondary metabolite pathways in myxobacteria. Numerous known or unknown gene clusters from various myxobacteria were cloned and electroporated into heterologous hosts, resulting in the expression and identification of desired products such as epothilone [88], myxothiazol [89], myxochromide [88], disorazol [90], cystobactamid [91], myxopyronins [92], and myxarylin [93].

Table 1
smBGCs in completely sequenced myxobacteria.

Strain	Genome size (Mb)	(G + C)%	Accession	Number of smBGCs	Proportion of smBGCs in chromosome
Minicystis rosea DSM 24000	16.04	69.1	NZ_CP016211.1	40	6.60%
Sorangium cellulosum So0157-2	14.78	72.1	NC_021658.1	35	9.60%
Sorangium cellulosum So ce836	14.60	72.0	NZ_CP012672.1	42	12.70%
Sorangium cellulosum So ce26	14.56	71.7	NZ_CP012673.1	36	10.90%
Archangium violaceum SDU8	13.21	68.9	NZ_CP069396.1	38	12.80%
Pyxidicoccus sp. SCPEA002	13.21	69.6	NZ_CP071090.1	32	12.40%
Sorangium cellulosum So ce 56	13.03	71.4	NC_010162.1	35	9.00%
Archangium violaceum SDU34	12.68	68.4	NZ_CP069338.1	35	9.60%
Archangium gephyra DSM 2261	12.49	69.4	NZ_CP011509.1	39	11.30%
Cystobacter fuscus DSM 52655	12.35	68.5	NZ_CP022098.1	47	12.00%
Labilithrix luteola DSM 27648	12.19	66.1	NZ_CP012333.1	12	3.10%
Chondromyces crocatus Cm c5	11.39	68.7	NZ_CP012159.1	33	12.90%
Sorangium cellulosum So ceGT47	11.26	72.6	NZ_CP012670.1	27	8.50%
Myxococcus xanthus 124B02	11.05	70.0	CP006003.1	29	9.60%
Myxococcus stipitatus DSM 14675	10.35	69.2	NC_020126.1	25	13.70%
Sandaracinus amylolyticus DSM 53668	10.33	72.0	NZ_CP011125.1	20	5.60%
Stigmatella aurantiaca DW4/3-1	10.26	67.5	NC_014623.1	35	12.10%
Corallococcus coralloides DSM 2259	10.08	69.9	NC_017030.1	31	13.10%
Melittangium boletus DSM 14713	9.91	68.4	NZ_CP022163.1	33	11.80%
Corallococcus coralloides B035	9.59	70.3	NZ_CP034669.1	32	15.30%
Myxococcus hansupus contaminant ex DSM 436	9.49	69.2	NZ_CP012109.1	28	12.40%
Haliangium ochraceum DSM 14365	9.45	69.5	NC_013440.1	21	8.10%
Corallococcus sp. EGB	9.43	70.4	CP079946.1	32	14.60%
Myxococcus xanthus KF4.3.9c1	9.43	68.9	NZ_CP017172.1	21	11.10%
Myxococcus xanthus DZ2	9.36	68.9	NZ_CP070500.1	22	12.50%
Myxococcus xanthus ATCC 27925	9.35	68.9	NZ_CP080534.1	22	12.60%
Myxococcus xanthus MC3.5.9c15	9.32	69.0	NZ_CP017174.1	22	11.50%
Myxococcus xanthus MC3.3.5c16	9.32	69.0	NZ_CP017173.1	22	11.50%
Myxococcus xanthus GH3.5.6c2	9.32	69.0	NZ_CP017169.1	21	11.20%
Myxococcus xanthus GH5.1.9c20	9.26	69.0	NZ_CP017170.1	21	11.30%
Myxococcus xanthus R31	9.25	68.9	NZ_CP068048.1	21	11.90%
Myxococcus xanthus DK1622::pDPO-Mxn116-Pvan-Tpase	9.20	68.9	NZ_CP065375.1	23	13.70%
Myxococcus xanthus DK 1622	9.14	68.9	NC_008095.1	22	12.80%
Myxococcus fulvus HW-1	9.00	70.6	NC_015711.1	23	11.40%
Corallococcus macrosporus DSM 14697	8.97	70.6	NZ_CP022203.1	22	10.80%
Myxococcus xanthus KF3.2.8c11	8.95	69.0	NZ_CP017171.1	24	13.50%
Anaeromyxobacter sp. Fw109-5	5.28	73.5	NC_009675.1	6	2.40%
Anaeromyxobacter sp. K	5.06	74.8	NC_011145.1	6	3.40%
Anaeromyxobacter dehalogenans 2CP-1	5.03	74.7	NC_011891.1	6	3.40%
Anaeromyxobacter dehalogenans 2CP-C	5.01	74.9	NC_007760.1	6	3.40%
Vulgatibacter incomptus DSM 27710	4.35	68.9	NZ_CP012332.1	3	2.30%

Table 2
Summary of the major advantages and limitations of genetic manipulation methods in myxobacteria.

Genetic manipulation method	Major components	Advantages	Limitations	Reference
Transduction	Phages	Applicable to any host that can be infected by phages	Phage-dependent operations are cumbersome and time-consuming	[70–73]
Transformation (electroporation)	Plasmids	Efficient transformation of large smBGCs	Not suitable for agglomerate myxobacteria	[81–83]
Conjugation	Mobilizable plasmid	The transfer process is stable and gentle	Inefficient and cumbersome	[101–105]
Homologous recombination	Homologous arms and homologous recombinases	Directed editing	The accuracy and efficiency are affected by homologous sequences	[111,113]
Site-specific recombination	Intergrase and integration sites of phage Mx8	Directed editing	The integration sites are limited, and integration efficiency decreases with the length of integrated fragments	[84,123,124]
Transposition	Transpon (Tn5, mariner)	Efficient insertion	Random insertion	[[136,140]
Plasmid	Shuttle vector pZJY41 derived from pMF1	Stable multi-copy expression of target genes	There is only one kind of shuttle plasmid applicable to limited hosts	[79,162]
CRISPR-Cas9 system	Cas9 protein and sgRNA	Directed editing or activation of target genes	The simplicity and efficiency need to be optimized; the endogenous CRISPR-Cas system remains to be developed	[96,159,160]

In addition to the model myxobacterium *M. xanthus*, the electroporation technique was also applied to *S. aurantiaca* DW4/3-1 [94], *S. cellulosum* So ce12 [95], So ce M4 [96,97], and *A. disciformis* An d48 [98]. One of the challenges of genetic manipulation in myxobacteria is the agglomerate growth characteristic, which severely restricts the uptake of external DNA as well as the screening and purification of mutant strains. Constructing dispersed-growing strains through mutation or domestication of wild-type strains effectively circumvents the genetic manipulation barrier. For instance, Zhang et al. obtained a completely dispersed growth mutant UV684 of *M. fulvus* HW-1 via ultraviolet mutagenesis. The mutant exhibits the same fruiting body formation and S-motility as the parent strain [99]. When the plasmid pMiniHimar1 was electrotransformed into UV684, the efficiency increased three to fivefold compared to HW-1. Notably, the model strain *M. xanthus* DK1622 is also a mutant

of the original strain FB and is capable of developing fruiting bodies on solid surfaces as well as growing dispersedly in liquid, thereby facilitating numerous genetic manipulations.

4.1.3. Conjugation

Conjugation is a well-understood process in which a donor bacterium transfers DNA to a recipient bacterium [100]. It is linked to the type IV secretion system (T4SS), where the pilus appendage acts as a needle, propelling relaxase across membrane barriers into the recipient, and the conjugative plasmid is replicated using the rolling-circle replication mechanism and pumped into the recipient cytoplasm with the available T4SS transport conduit [101]. Although tedious, conjugation is a gentle and stable method for DNA transfer. Subsequently, using a mobilization plasmid, the recombinant conjugative plasmid carrying the target gene can be transferred from an *E. coli* donor strain to a myxobacterial recipient strain, where it can replicate autonomously or be incorporated into the genome.

The conjugation system has been utilized effectively in *M. xanthus* [102], *S. aurantiaca* [103], and *S. cellulosum* [104,105] to characterize the biosynthetic genes for secondary metabolites such as soraphen, epothilone, and chivosazole. It was reported that the conjugation efficiency of *S. cellulosum* increased as the length of the homologous arm increased [105]. Julien et al. cloned a mariner-based transposon into the conjugation plasmid in 2003, causing the plasmid to randomly insert into the genome of So ce90 following conjugation[107]. This conjugation system was applicable to So ce90 but not to other *Sorangium* strains, e.g., So ce12. Kopp et al. developed a method for DNA transfer from *E. coli* to So ce56 and So ce12 via conjugation using biparental or triparental mating and obtained transposon mutants with an inactive chivosazole biosynthetic gene cluster[108]. In addition to establishing the conjugative system in So0157-2 and So02007-3, Xia et al. found that the addition of low-dose antibiotics to the conjugation medium significantly increased conjugation efficiency [107]. Generally, the conjugation system developed for an *S. cellulosum* strain may be inapplicable to others, including strains of the same species [106], and no universal conjugation system has yet been established for different *S. cellulosum* strains, let alone other myxobacteria. The extraction of bioactive natural products from *Sorangium* strains might depend more on the heterologous expression of smBGCs than on the genetic performance of native producers.

4.2. Gene editing tools in myxobacteria

After transfer, the stable existence of extracellular DNA in host cells is dependent upon either autonomous plasmid replication or integration into the host genome. Homologous recombination between the host genome and homologous arms allows suicide plasmid or homologous fragment integration [108]. Typically derived from phages, integrases catalyze the site-specific recombination of foreign DNA with the genome at attachment sites [109]. Unlike site-specific insertion based on recombination and integration, during transposition, exogenous DNA is randomly inserted into the genome [112]. For myxobacteria, unless an autonomously replicating plasmid is available, gene editing studies in myxobacteria are carried out primarily via integration mediated by recombinases or integrases[79]. Myxobacteria have been introduced to the burgeoning clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology in recent years.

4.2.1. Homologous recombination

Through homologous recombination, a suicide plasmid carrying a fragment homologous to the host chromosome will be incorporated into the chromosome once introduced. Cho and Zusman once created a plasmid library containing approximately 500-bp random DNA fragments of *M. xanthus*. These fragments were electroporated into wild-type *M. xanthus* cells and integrated into the chromosome by homologous recombination, thereby producing a random pool of *M. xanthus* insertion mutants [111]. The screening of insertion mutants revealed two essential genetic regions for the production of EPS: the EPS synthesis region and the EPS-associated region [112]. The integration will result from the functional inactivation of the inserted gene or functional complementation of a gene carried by the plasmid. In order to avoid affecting the expression of in situ genes, it is necessary to choose an appropriate recombination site. For instance, the 3′ end of the *dev* operon was once chosen as the recombination site for the heterologous expression of the epothilone gene cluster in *M. xanthus* [85].

Based on homologous recombination, a knockout system based on the pBJ113 plasmid [113] containing the kanamycin resistance (Km^r)-galactosidase kinase (*galK*) gene cassette (KG) has been developed and is widely used for traceless gene editing in *M. xanthus* [114–117]. Km^r is used as a positive selection marker for the first round of homologous recombination, and *galK* from *E. coli* makes cells sensitive to 1% galactose for isolating mutants that have lost *galK* in the second round of homologous recombination. If the second recombination occurs between the same homologous arms as the initial recombination, the strain will revert to its wild-type state; otherwise, the target gene is deleted completely. This double-crossover method was once used to determine the necessity of *groEL2*, but not *groEL1*, for the biosynthesis of the secondary metabolite myxovirescin in *M. xanthus* DK1622 [117,118]. Similar methods have been used to identify transcriptional regulators of secondary metabolites, primarily in *M. xanthus*, including specific regulators for the biosynthesis of epothilone [119] or carotenoid [120], as well as some global regulators [121,122]. Knockout of *epoF*, the last gene of the epothilone gene cluster, caused the conversion of epothilones A and B to epothilones C and D, indicating that the gene is responsible for the formation of the epoxide at C12-C13 [85].

Exploring the biosynthetic pathways of smBGC via the traceless mutation is also very encouraging. Because there are numerous similar smBGCs in a myxobacterial strain and highly similar sequences in smBGCs such as PKS and NRPS, homologous recombination-based gene editing is unavoidably time-consuming and inefficient. Moreover, using the two-round schedule in *M. xanthus*, homologous recombination, mutant screening, and purification of a traceless knockout strain typically require at least 1 month.

4.2.2. Site-specific recombination

In *M. xanthus*, the site-specific recombination system mediated by a myxophage-derived recombinase is also effective in enhancing genetic performance. Mx8 has been completely sequenced and is the myxophage that is best understood. Mx8 integrates into the host genome as a stable prophage via site-specific recombination between the phage *attP* site and *attB1* or *attB2*, two neighboring attachment sites in the host chromosome [123]. Julien et al. characterized the integrase gene and *attP* site in Mx8 and the insertion sites in *M. xanthus* chromosome [124]. A *lacZ* reporter gene integrated at the Mx9 *attB* site demonstrated a higher transcriptional level than the same integration at the Mx8 *attB* site. Approximately 300-fold more site-specific recombination occurred at the chromosomal *attB* site than at the native *lonD* locus in *M. xanthus* [125]. In *M. xanthus*, the phage attachment sites facilitate the efficient and stable expression of exogenous genes or additional copies of existing genes.

Salmi et al. cloned the *attP* site and the integrase coding gene into the *E. coli* plasmid pBGS18, constructing the plasmid pPLH343, which cannot replicate in *M. xanthus* cells but can insert into the *attB* site of the *M. xanthus* genome [126,127]. Similar plasmids such as pSWU19 (Km^r), pSWU30 (Tetracycline-resistant, Tet^r), and pCK T7A1 att (Km^r) were constructed and are currently widely used in *M. xanthus* for expression and functional verification of genes associated with primary metabolism [128], motility [2,129], predation [130], development [131], and self-recognition [4]. Gross et al. modified and chemically synthesized the cystobactamid biosynthetic gene cluster (about 58 kb) of *Cystobacter velatus* Cbv34 in fragments, which were then assembled and integrated into the *M. xanthus* DK1622 genome via the Mx8 integrase [91]. According to Zhu et al., however, the recombination efficiency of the Mx8 *attB*

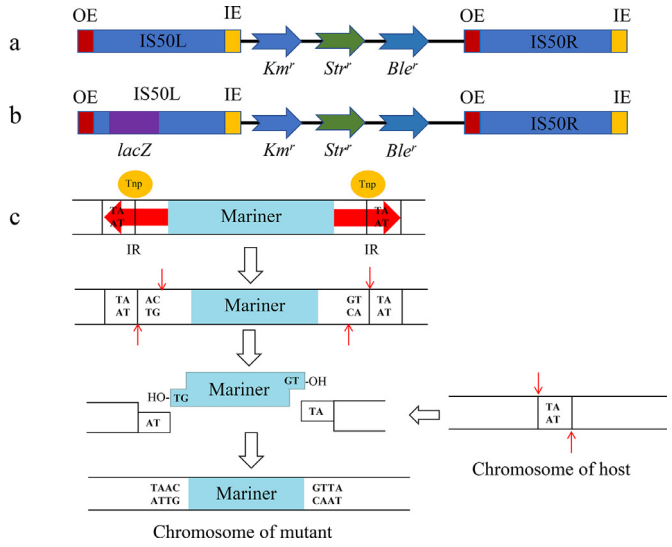

Fig. 4. Transposons widely used in myxobacteria. a, Tn5 transposon; b, mutant Tn5-*lacZ* transposon; c, Mariner transposon and insertion mechanism. IS50L and IS50R are two inverted insertion sequences flanking a transposase gene; OE and IE, 19-bp reverse sequence recognized by transposase at both ends of IS50L and IS50R. Tnp, transposase; IR, Tnp-recognized inverted repeat sequence. A thin red arrow slicing through Tnp. *Km*^{r}, kanamycin-resistant gene; *Str*^{r}, streptomycin-resistant gene; *Ble*^{r}, bleomycin-resistant gene; *lacZ*, galactosidase gene.

site decreased sharply as the integration fragment size increased, and site-specific recombination at the *attB* site is generally not preferred for the expression of smBGCs larger than 50 kb [84].

In addition to efficiently transporting large smBGCs, these suicide plasmids effectively identify genes involved in the synthesis of natural products by complementing or overexpressing target genes. Using the Pck-T7A1-att plasmid, Volz et al. identified two bacterial enhancer-binding proteins (bEBPs) in *M. xanthus* that functioned as direct regulators to activate or inhibit various secondary metabolites [121]. Subsequently, using plasmid pSWU30 to overexpress the *esi* gene upstream of the epothilone gene cluster, Yue et al. determined that this gene encodes a transcription factor that inhibits the transcription of the epothilone gene cluster specifically [119]. Integrated expression based on Mx8 site-specific integration remains one of the most widely used and efficient methods for gene expression in myxobacteria.

4.2.3. Transposition

Transposons are genetic elements that can "jump" to various genome locations within a cell. Tn5 is an *E. coli* transposon made up of a sequence encoding three antibiotic resistance genes (kanamycin, bleomycin, and streptomycin) and a Tn5 transposase Tnp gene, flanked by two inverted insertion sequences, IS50L and IS50R (Fig. 4a) [132]. Each of the two IS50 sequences has a pair of 19-bp reverse sequences at both ends, outside ends (OEs) and inside ends (IEs). Tnp recognizes and cleaves the OE sequence to liberate Tn5, which then binds and inserts randomly into the host genome [133]. The random insertion characteristic with the antibiotic selection marker is convenient for subsequent phenotypic screening, genetic mapping, or clonal identification, making the Tn5 system a highly productive DNA transposon [134].

Tn5 is the first transposable element used in myxobacteria, and it continues to be widely employed in the construction of insertion mutants in *M. xanthus*. Initially, Tn5 was introduced into *M. xanthus* via phage transfection. Following the transduction of the *E. coli* phage P1::Tn5 into *M. xanthus*, Tn5 was randomly inserted into the genome, producing stable kanamycin-resistant mutants [71]. Once Tn5, along with the antibiotic resistance gene, is integrated into the host genome, the likelihood of this element being transposed to other sites is less than 1/1000 [135]. Therefore, P1::Tn5 is predisposed to generate mutants with a single Tn5 insertion, which facilitates the identification of mutant genes responsible for phenotypic changes. Based on Tn5, Sasakawa & Yoshikawa constructed a series of Tn5 transposon variants by replacing the selection marker with various antibiotic resistance genes (including resistance genes against tetracycline, chloramphenicol, gentamicin, trimethoprim, streptomycin, and ampicillin). These variants provide more options for various scenes[138]. Additionally, the authors created a temperature-sensitive suicide plasmid R388 as a vector for transporting Tn5 elements into various hosts. Kroos et al. replaced a portion of IS50L with the lacZ gene lacking a promoter and constructed the Tn5-*lacZ* cassette [76] (refer to Fig. 4b). When P1::Tn5-*lacZ* is transduced into *M. xanthus, lacZ* will be co-transcribed with the inserted gene if the Tn5-*lacZ* gene was inserted into a transcription unit on the host chromosome in the same orientation, which can be detected by β-galactosidase activity [137–140].

The Mini–Himar element (magellan-4) is a derivative of the mariner transposon superfamily from eukaryotes and is frequently used in myxobacteria [141]. The Mini–Himar element integrates into the chromosome through a straightforward mechanism of cut-and-paste. The transposase cleaves DNA at the inverted repeat (IR) sequences that mark each transposon end and then insert the excised transposon at the dinucleotide TA site (Fig. 4c) [142]. In contrast to other transposons, the mariner's transposition is dependent solely on the self-transposase. This allows the mariner to be transferred horizontally between species, making it an attractive tool for genetic engineering [143]. Youderian et al. (2003) reported that Mini–Himar is active in *M. xanthus* and generates more extensive insertions than bacterial transposons, including Tn5 [140]. The authors identified 30 new genes required for A motility using the protocol. This transposon has been utilized since then to analyze and identify genes in *M. xanthus* [144,145]. The mariner-based transposon was also developed and introduced into *S. cellulosum* strains via conjugation; similarly, the transposon could randomly insert into the chromosomes of So ce90 and So ce12 [146].

Random insertion makes Tn5 and its variants useful for identifying and validating the coding or regulatory genes for secondary metabolites. Varon et al. reported that the transposition of Tn5-lacZ and TnV (another Tn5 derivative containing the replication origin of plasmid pSC101) into *M. xanthus* resulted in 8,381 kanamycin-resistant mutants, of which 24 mutants were nonproducers, and three produced higher levels of TA than the parent strain [147]. There was a co-transduction between kanamycin resistance and the altered TA phenotype in most mutant strains. The gene cluster for the biosynthesis of the heterocyclic quinone antibiotic saframycin Mx1 was inactivated and tagged by Tn5 insertions in *M. xanthus* DM504/15, and the tagged genes were cloned and identified, thereby identifying the genomic region involved in saframycin biosynthesis [148]. Isolation and genetic analysis of *M. xanthus* mutants generated by Tn5 insertions helped identify the key genetic loci involved in light-induced carotenogenesis and elucidate the molecular mechanisms underlying the light-dependent signaling and regulation of the transcriptional response leading to carotenoid biosynthesis [120,149].

In addition, since transposition-mediated gene integration is not constrained by fragment size, transposable elements are a useful tool for the transfer of large smBGCs. For example, Fu et al. achieved heterologous expression of epothilone by introducing the epothilone biosynthesis gene cluster derived from *S. cellulosum* So ce90 into *M. xanthus* DK1622 with the aid of the Mini–Himar element[88]. The biosynthetic gene cluster of disorazol, a macrocyclic polyketide produced by *S. cellulosum* So ce12, was identified by transposon mutagenesis with Mini–Himar [95] and cloned in a bacterial artificial chromosome library on the basis of which the 58-kb core gene cluster was reconstituted by Red/ET recombineering and expressed in *M. xanthus* DK1622 [90]. Zhu et al. also utilized the Mini–Himar method to randomly insert the epothilone gene cluster of *S. cellulosum* So0157-2 into *M. xanthus* DZ2, and obtained heterologous expression mutants of epothilone with varying production capacities [84]. They discovered that the transposon

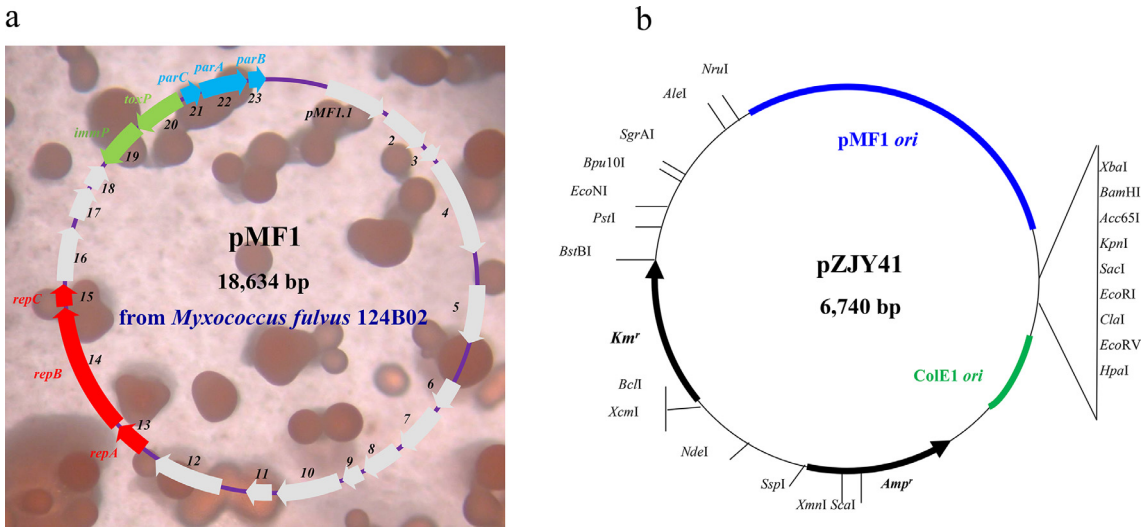

Fig. 5. pMF1 plasmid from *M. fulvus* 124B02 (a) and the shuttle vector pZJY41 (b). *parCAB*, par operon for the partitioning of pMF1, related genes were highlighted with blue arrows; *repABC*, operon encoding the replication proteins, related genes were highlighted with red arrows; *taxP* and *immP*, a pair of nuclease toxins and immune protein expressed by pMF1, were highlighted with corresponding green and blue arrows, respectively. Other genes in pMF1 with uncertain functions were labeled with gray arrows. pMF1 ori, a replicon of pMF1, responsible for the replication of pZJY41 in *M. xanthus*; ColE1 *ori*, replicon responsible for the replication of pZJY41 in *E. coli*.

efficiently integrated large fragments into the host genome and that the insertion sites substantially affected the expression of foreign genes.

4.2.4. Expression vectors

All of the abovementioned plasmids used in the genetic manipulation of myxobacteria must integrate into the host genome via homologous recombination, transposition, or site-specific integration after entering the host, and the self-replicating plasmid from myxobacteria was not reported until 2008. Zhao et al. screened 150 myxobacterial strains and reported pMF1, the first circular plasmid capable of self-replication, from *M. fulvus* 124B02 [79] (Fig. 5a). This plasmid is 18,634 bp long, contains a G+C content of 68.7%, containing 23 open-reading frames. In the repABC operon consisting of pMF1.13-pMF1.15 (Fig. 5a), pMF1.14 encodes an essential replication initiation protein with abundant disordered regions but devoid of DNA/protein binding motifs [79,150]. It is hypothesized that the extremely restricted host range of the pMF1 replicon in *Myxococcus* cells is due to its unmatched characteristics. Further research revealed that the pMF1.21-pMF1.23 operon (labeled parCAB in Fig. 5a) was responsible for the partitioning of pMF1 [151] and that the natural expression of parC positioned upstream of parA was essential for the plasmid inheritance in *Myxococcus* cells [152]. In addition, pMF1.20 and pMF1.19 encode a pair of nuclease toxins and immune protein that function as a post-segregational killing system, which has been proposed to aid in the maintenance of plasmid PMF1 in *M. fulvus* cells [153]. The other predicted genes in pMF1 either have homologs in sequenced myxobacterial genomes or lack homology to any known sequence [154].

The *Myxococcus* plasmid pMF1, which replicates autonomously, provides a new tool for genetic manipulation. Based on the pMF1 replicon, vectors such as pZJY41 and pZJY156 were designed to shuttle expression between *M. xanthus* and *E. coli* [79]. The plasmid pZJY41 contains the pMF1 *ori*, the high-copy-number ColE1 *ori*, two antibiotic resistance genes as selection markers, and a region with multiple clone sites (MCS) (Fig. 5b). The target gene cloned in the MCS of pZJY41 will express 10–20 copies of the plasmid in *M. xanthus* DK1622, resulting in overexpression [155–158]. In addition to the expression and identification of targeted genes, pZJY41 is also used to express small guide RNA (sgRNA) to mediate gene editing with CRISPR and CRISPR-associated protein 9 (Cas9) system [159] or to promote the transcription of epothilone biosynthesis with the sgRNA recruited RNAP in *M. xanthus* [160,161].

In 2021, Panter et al. reported the presence of the second self-replicating plasmid pSa001 in *Sandaracinus sp.* MSr10575 [162]. The 209.7-kb pSa001 contained a large PKS–NRPS hybrid gene cluster that was activated by homologous recombination mediated exchange of the native promoter and produced antitumor sandarazols. The replication and division mechanisms of this plasmid require additional research.

4.2.5. CRISPR-Cas system

Numerous prokaryotic organisms employ CRISPR-Cas systems to repel mobile genetic elements [163]. The discovery of the CRISPR-Cas system and its transformation into a potent gene editing tool have revolutionized the field of molecular biology [164–166]. The widespread use of the CRISPR-Cas9 system in both prokaryotes and eukaryotes is due to its simplicity and flexibility. This system provides a robust and multiplexable platform for genome editing, transcriptional perturbation, epigenetic modulation, and genome imaging [167,168]. CRISPR-Cas9-based genome engineering has also been utilized for the discovery of natural products in various bacteria, fungi, and plants [169].

In 2018, Peng et al. constructed the CRISPR-based activation system in myxobacteria to promote the biosynthesis of secondary metabolites for the first time [160]. A codon-optimized, nuclease-dead Cas9 (dCas9) was fused with various activator proteins and introduced into the epothilone-producing producer *M. xanthus* ZE9. With the expression of sgRNA targeting the promoter of the epothilone operon, the dCas9-activator proteins recruited RNAP to promote the transcription of the epothilone genes, resulting in a 1.5-fold increase in epothilone yield. This CRISPR-dCas9 system was then used to individually or collectively activate internal promoters in the epothilone operon, revealing the multiple interferences between promoters that altered the transcriptional profile of operon genes and the production of epothilones [161]. Similarly, Ye et al. introduced the CRISPR-dCas9 system into the *S. cellulosum* strain So ce M4 to activate the transcription of the epothilone gene cluster using VP4 as the activator [97]. It was also determined that the CRISPR-Cas9 system is effective for the deletion of large genome fragments in *M. xanthus* DK1622 [159]. According to the protocol, the *Cas9* gene and sgRNA were integrated into the host chromosome via site-specific recombination. A temporally high expression strategy was employed to reduce cell damage caused by high Cas9 expression, and a tRNA-sgRNA-tRNA chimeric structure was designed to ensure correct targeting by sgRNA sequence. Additionally, 14% of the 92-kb smBGC

for myxalamids was deleted using the strategies, demonstrating the application potentials of CRISPR-Cas9 in the genome rational design and deep modification of *M. xanthus*.

In spite of its applicability, the CRISPR-Cas9 system in *M. xanthus* requires further optimization to make its operation easier and more efficient. For instance, independently introduced sgRNA, not in conjunction with Cas9, will make it possible to edit different sites or simultaneously implement multi-site editing. The introduction of a non-homologous end-joining system (NHEJ) into *M. xanthus* may omit the need for homologous arm introduction. Notably, *M. xanthus* DK1622 possesses three endogenous CRISPR-Cas systems, MxI-B, I-C, and III-B [170], which are being identified and developed as effective gene editing tools (unpublished). For the discovery of natural products in myxobacteria, ever-more-effective CRISPR-Cas systems are anticipated to be utilized.

5. Engineering of secondary metabolism in myxobacteria

The social characteristics, indistinct genetic backgrounds, and limited genetic manipulation techniques make it very difficult to directly modify smBGCs in their original myxobacterial producers. Heterologous expression is an alternative solution to this issue.*M. xanthus* DK1622 is the model strain of myxobacteria and is an ideal host for studying and expressing the secondary metabolites of myxobacteria, which are typically expressed poorly in other microbial hosts [171]. Among numerous myxobacterial metabolites, the heterologous expression of epothilones is currently the most thoroughly investigated. Epothilones are 16-member macrolides that were first isolated from *S. cellulosum* So ce90 and exhibit antifungal and cytotoxic properties [36,172]. Epothilones function as antineoplastics similarly to paclitaxel. They bind to microtubules to prevent the formation of spindles during mitosis, causing cancer cells to undergo apoptosis [173,174]. Moreover, they have therapeutic potential in the treatment of neurodegeneration and nervous system injury [51,52]. In myxobacteria research, heterologous expression and biosynthesis regulation of epothilones are among the hottest topics due to the low productivity and limited modification options of the original producers.

5.1. Biosynthesis and regulation of epothilone

In 2000, the biosynthetic gene cluster and biosynthetic pathway of epothilones were identified and deduced [175,176]. As depicted in Fig. 6, the 56-kb gene cluster consists of six multifunctional genes in the same transcription direction (*epoA, epoP, epoB, epoC, epoD, and epoE*) and a P450 gene *epoF* (also *epoK* in some publications), encoding five PKS (EPOSA, EPOSB, EPOSC, EPOSD, and EPOSE), an NRPS (EPOSP) and a cytochrome P450 epoxidation enzyme (EPOSF) for the elongation and processing of epothilones. Each PKS protein is comprised of one or more modules containing discrete domains, including the indispensable β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, and the dispensable modification domains such as ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), methyltransferase (MT) or thioesterase (TE), whereas EPOSP consists of condensation domain (C), a peptidyl carrier protein (PCP), adenylation domain (A), and an oxidation domain (OX). The synthetic pathway begins with the synthesis of a thiazole ring, followed by eight steps for the polyketide chain extension, the cyclic release of the polyketide chain, and the epoxidation of epothilone C/D to epothilone A/B [85].

Zhu et al. characterized the promoters of epothilone gene clusters from various producers and discovered that the expression of all operons is regulated by a dual promoter P_{epo} (with two −35 and −10 regions and two distinct TSSs), a hairpin structure, and the distal non-coding region between the promoter and operon [177] (Fig. 6). Simple replacement of epothilone promoter P_{epo} with strong endogenous promoters (P_{pilA}, P_{groEL}) did not improve epothilone synthesis in *M. xanthus*, most likely due to inconsistency in the transcriptional temporal modes between nominated promoters and those of secondary metabolite synthesis [178]. Nonetheless, the multi-copied P_{epo} tandem substantially increased the transcription level of epothilone genes, resulting in a remarkable increase in epothilone yield. A gene located directly upstream of *epoA* in *S. cellulosum* So0157-2 encodes the negative transcriptional regulator Esi, which binds to the hairpin region and inhibits the transcription of epothilone genes; inactivation of *esi* in So0157-2 or in heterologous host ZE10 increased the yield of epothilones by 4.1-fold and 38%, respectively [119] (Fig. 6). This is the first transcriptional regulator of epothilone biosynthesis that has been identified. In addition to the promoter in front of the epothilone gene operon (P_{epo}), multiple internal promoters were identified within the operon. Activation of the P_{epo} and/or internal promoters with the CRISPR-dCas9 activation system promoted the epothilone synthesis, demonstrating the complex regulation of epothilone expression [160,161] (Fig. 6). Notably, aided by high-throughput evaluation of mutants, the epothilone production in *S. cellulosum* So0157-2 could be significantly enhanced by a combination of UV mutation, recursive protoplast fusion, and genome shuffling [179]. However, the underlying mechanism remains unclear. Additional research into the transcriptional regulatory mechanism will inform the genetic modification of producing strains to optimize epothilone production.

5.2. Heterologous expression of epothilone biosynthetic gene cluster

Tang et al. (2000) introduced the epothilone biosynthesis gene cluster from *S. cellulosum* SMP44 into *Streptomyces coelicolor* CH999, and epothilone levels reached 50–100 μg/L [180]. In vivo knockout of *epoF* and in vitro assays with recombinant epoF expressed in *E. coli* demonstrated the function of EPOSF. Other *Streptomyces* strains, such as *S. venezuelae* DHS2001 [181] and *S. lividans* ZX7 [182], were also used to express epothilone with various applicable promoters, but yields were either undetectable or extremely low. In *S. venezuelae*, overexpression of *pikD*, a positive regulatory gene of the endogenous pikromycin biosynthetic gene cluster, increased epothilone production. Two adjacent genes of the epothilone gene cluster, orf3 and orf14, were identified as putative transport genes because their deletion resulted in a threefold decrease in epothilone D production. Also redesigned and synthesized was the entire gene cluster, which expressed as soluble proteins in *E. coli* K431-37-2A and produced trace amounts of epothilones C and D (< 1 μg/L) [183]. In addition, cells expressing the *epoC-epoD-epoE* genes synthesized epothilones C and D when fed a thioester of the normal substrate for epoC, demonstrating the composability of the epothilone synthetic pathway. Bian et al. (2017) expressed the entire epothilone gene cluster from *S. cellulosum* So ce90 in *Schlegelella brevitalea* DSM 7029. Host-specific medium optimization and genetic modification (introduction of the biosynthetic pathway for precursors and overexpression of rare tRNA genes) significantly increased epothilone production in *S. brevitalea* [184]. The epothilone gene cluster of *S. cellulosum* So0157-2 was similarly expressed in *S. brevitalea* DSM 7029, resulting in an initial yield of 80 μg/L, which was increased by 7.5-fold through the reconstruction of the methylmalonyl-CoA biosynthetic pathway to improve precursor supply [185]. The authors reassembled the mega epothilone gene cluster using BioBricks and SSRTA techniques, thereby creating an artificial pathway in which each gene was transcribed with an independent promoter to regulate the pathway's equilibrium and maximize its activity. Further addition of sucrose debottlenecked the DSM 7029 strain, which improved epothilone production by enhancing the potentials of the optimally reassembled gene cluster; the epothilone yield finally reached 82 mg/L.

To date, the epothilone gene cluster has been expressed in various hosts (genetic modifications of the epothilone gene cluster for heterologous expression are depicted in Fig. 7), among which the model species *M. xanthus* possesses the most promising properties (Table 3). Julien et al. inserted the epothilone gene cluster into the *M. xanthus* DZ1 genome by means of homologous recombination. The initial yield

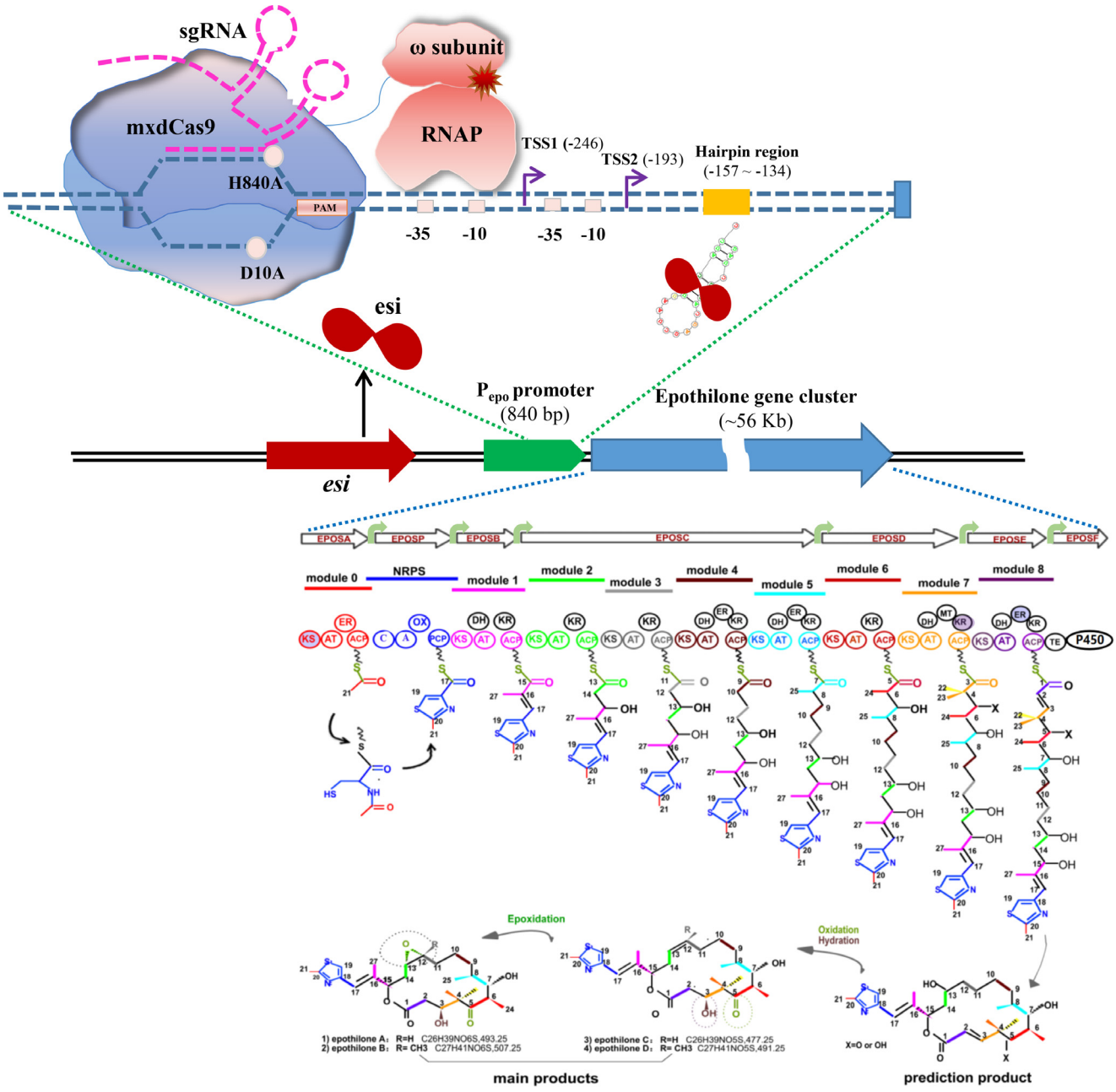

Fig. 6. The gene cluster and biosynthesis pathway of epothilone (modified from reference 178), as well as investigations into its regulatory mechanisms. KS denotes the β-ketoacyl synthase domain; AT, the acyltransferase domain; ACP, the acyl carrier protein domain; KR, the ketoreductase domain; DH, the dehydratase domain; ER, the enoyl reductase domain; MT, the methyltransferase domain; TE, the thioesterase domain; C, the condensation domain; PCP, peptidyl carrier protein; A, adenylation domain; OX, oxidation domain. The shaded domains are inactive in the biosynthesis pathway. TSS, transcriptional start site; the base position of the TSSs and hairpin region was defined with reference to the translation start site as +1. esi, negative transcriptional regulator bing to the hairpin region of P_{epo}. H840A and D10A were two mutant amino acids in dCas9. Green arrows indicate the internal promoters of the epothilone gene cluster.

of epothilone A and epothilone B in mutant strains was ~100 μg/L, whereas the removal of *epoF* increased the yield of epothilone D to 200–400 μg/L [85]. Using Red/ET recombination, Fu et al. stitched the full-length epothilone gene cluster into a single plasmid carrying the magellan-4 transposon, which mediated the integration of epothilone gene cluster into the genomes of *M. xanthus* DK1622 and *Pseudomonas putida* FG2005, respectively [88]. This study demonstrated that transposons can efficiently deliver large transgenes to heterologous hosts. The mutants derived from *M. xanthus* DK1622 synthesized ~100 μg/L of epothilones, whereas none was detected in the *P. putida* mutants. The epothilone gene cluster of *S. cellulosum* So0157-2 was introduced into various *M. xanthus* (*M. xanthus* DZ2, DK1622, and SW504) by a mariner transposon, and allopatric integrations in genome significantly altered host transcriptomes, resulting in a range of epothilone production capacities (0.1–1000 μg/L) [84]. The 1 mg/L production represents the highest initial yield of heterologous expression of epothilones, and the different yields resulting from genomic position effects suggest that by screening more mutants, more productive strains can be anticipated.

In general, compared to other heterologous expression hosts, *M. xanthus* exhibited superior productivity of epothilones, and heterologous expression of epothilones in *M. xanthus* resulted in relatively higher initial yields. *M. xanthus*, unlike *E. coli* and *Burkholderia*, produces diverse PKS/NRPS secondary metabolites, such as myxovirescin, myxalamid, DKxanthene, and myxochromide, which are constructed sim-

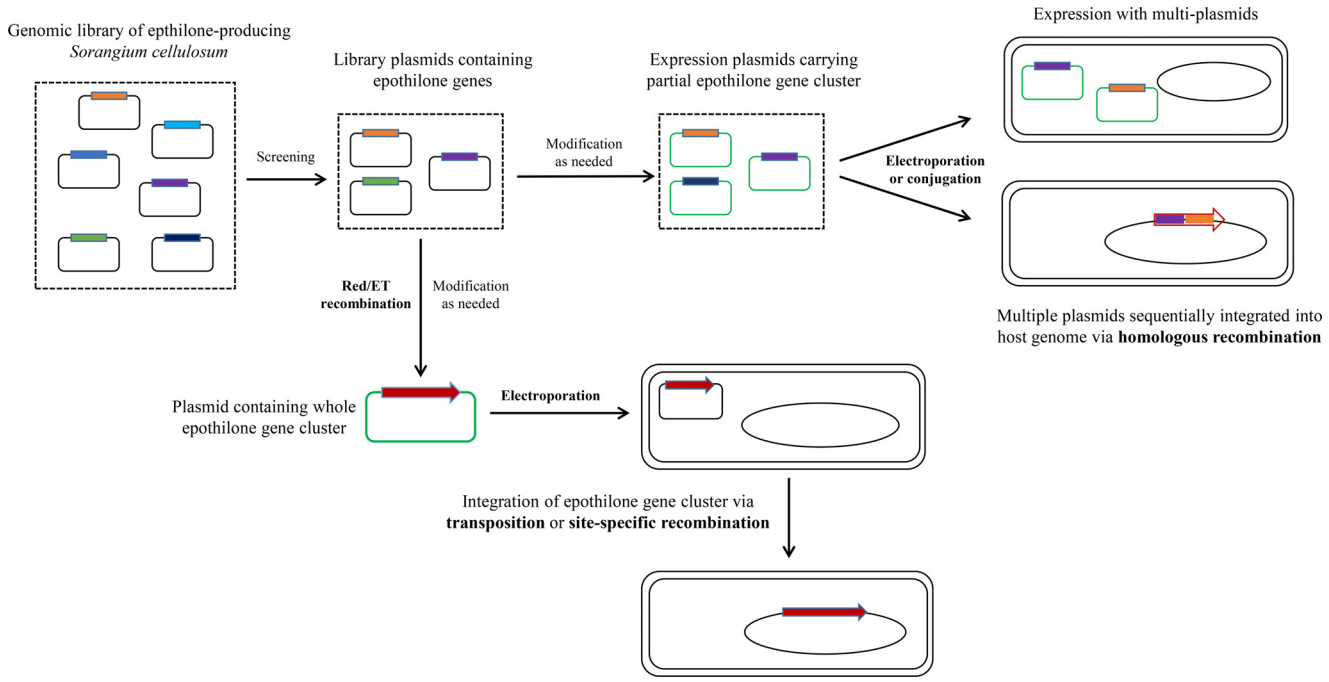

Fig. 7. Genetic modification of the epothilone gene cluster for heterologous expression. Library plasmids containing partial epothilone gene cluster are screened from the genomic library of epothilone-producing *Sorangium cellulosum*. After customized modifications (such replacement of suitable promoter and addition of necessary components like transposon), plasmid expression is introduced into the host for extranuclear or integrated expression. Alternatively, the library plasmids are modified with Red/ET to assemble the entire epothilone gene cluster, and the resulting expression plasmids are introduced into the host and then integrated into the genome through transposition or site-specific recombination. Red arrow, whole epothilone gene cluster; colored rectangles, various epothilone gene cluster components; black rounded rectangle, skeleton of common plasmid; green rounded rectangle, skeleton of expression plasmid; black oval, host genome.

Table 3
Heterologous expression of the epothilone biosynthetic gene cluster in different hosts.

Expression host	Origin of gene cluster	Expression form	Initial yield	Optimized method	Optimized yield	Reference
S. coelicolor CH999	*S. cellulosum* SMP44	Expressed by plasmids	50–100 μg/L	-	-	[180]
S. venezuelae DHS2001	*S. cellulosum* So ce90	Expressed by plasmids	0.1 μg/L	Knockout of *epoD*	0.4 μg/L	[181]
E. coli K431-37-2A	Chemical synthesized	Expressed by plasmids	< 1 μg/L	-	-	[183]
P. putida FG2005	*S. cellulosum* So ce90	Integrated via transposition	Undetectable	-	-	[86]
M. xanthus DZ1	*S. cellulosum* So ce90	Integrated via homologous recombination	200–400 μg/L	Addition of resin and methyl oleate	85 mg/L (Fed-batch fermentation)	[112,188,189]
M. xanthus DK1622	*S. cellulosum* So ce90	Integrated via transposition	100 μg/L	-	-	[86]
M. xanthus DK1622	*S. cellulosum* So ce90	Integrated via site-specific recombination	100 μg/L	-	-	[187]
M. xanthus DK1622, DZ2, SW504	*S. cellulosum* So0157-2	Integrated via transposition	0.1–1000 μg/L	Addition of resin and methyl oleate; knockout of negative transcriptional regulator; CRISPR-activation	28 mg/L	[84,119,160,178]
S. brevitalea DSM 7029	*S. cellulosum* So ce90	Integrated via transposition	4 μg/L	Medium Optimization; enhancement of precursor supply; overexpression of rare tRNA genes	307 μg/L	[184]
S. brevitalea DSM 7029	*S. cellulosum* So0157-2	Integrated via site-specific recombination	80 μg/L	Enhancement of precursor supply; reassembling of gene cluster; debottlenecking of the cell autolysis	82 mg/L	[185]

ilarly from malonyl-CoA or methylmalonyl-CoA [34]. Engineering of pathway-specific regulators [121] or enzymes responsible for posttranslational activation of natural product assembly lines may increase the total amount of the abovementioned compounds [186], which implies that the metabolic pathways of *M. xanthus* can provide abundant precursors for the synthesis of epothilones without special metabolic pathway modification. However, despite the fact that competition for precursor monomers was not regarded as the primary constraint on epothilone production [187], engineering or feeding monomers will increase production yields. In 2014, Osswald et al. redesigned and reassembled the epothilone gene cluster by codon optimization and introduction of unique restriction sites to permit pathway assembly and future interchangeability of modular building blocks from the epothilone megasynthetase [187] and the successful heterologous production of epothilones unambiguously demonstrated the functionality of the artificial pathway in *M. xanthus*. This research promises future engineering of epothilone biosynthesis and production optimization through a highly adaptable assembly strategy.

Resin XAD-16 can absorb epothilone products [36], whereas methyl oleate increases cell density and epothilone yield [85]. Combining. XAD-

Table 4
smBGCs and GIs in *M. xanthus* DK1622.

smBGC	Start	End	Length (bp)	Type	Products
cluster 1	1015682	1031120	15438	Terpene	Carotenoid
cluster 2	1490426	1537319	46893	NRPS	
cluster 3	1771438	1813469	42031	NRPS	
cluster 4	1863446	1927180	63734	NRPS	
cluster 5	2778046	2800369	22323	Thioamitides	
cluster 6	3235611	3284053	48442	NRPS/Type I PKS	
cluster 7	3318246	3340534	22288	Lanthipeptide-claaa-II	
cluster 8	4013494	4058514	45020	Type I PKS	Myxalamid
cluster 9	4135538	4145774	10236	RiPP	
cluster 10	4202141	4363714	161573	NRPS/Type I PKS	Myxochelin
cluster 11	4432172	4452468	20296	RRE-containing	
cluster 12	4481594	4563638	82044	NRPS/Type I PKS	Myxoprincomide
cluster 13	4736846	4822556	85710	PKS/NRPS	Myxovericin
cluster 14	4885909	4923967	38058	NRPS/Type I PKS	
cluster 15	4977639	5044926	67287	NRPS/Type I PKS	Myxochromide
cluster 16	5105157	5116326	11169	RiPP	
cluster 17	5235101	5311872	76771	NRPS/Type I PKS	Dkxanthene
cluster 18	5387898	5488693	100795	NRPS/Type I PKS	
cluster 19	5585364	5671886	86522	NRPS/Type I PKS	
cluster 20	5715538	5794912	79374	NRPS/RiPP	
cluster 21	6157224	6205340	48116	Thiopeptide/RiPP	
cluster 22	7706986	7729253	22267	Terpene	Cittilin
cluster 23	7855962	7897173	41211	Ladderane/lanthipeptide-class-II	
cluster 24	8147550	8188632	41082	T3PKS	Alkylpyrone
GI1	148705	177189	28485	Containing mobile element	
GI2	660595	676929	16335	Containing mobile element	
GI3	1402684	1448711	46028	Containing mobile element	
GI4	1543126	1569486	26361	Containing mobile element	
GI5	2013057	2037294	24238	Containing mobile element	
GI6	2080781	2095849	15069	Containing mobile element	
GI7	2107749	2127300	19552	Containing mobile element	
GI8	2179596	2204689	25094	Containing mobile element	
GI9	2216216	2242401	26186	Adjacent to tRNA gene	
GI10	2427372	2460798	33427	Containing mobile element	
GI11	3290237	3299422	9186	Adjacent to tRNA gene	
GI12	4192698	4217512	24815	Adjacent to tRNA gene	
GI13	5059847	5063412	3566	Containing mobile element	
GI14	5066197	5081299	15103	Containing mobile element	
GI15	5532695	5545491	12797	Adjacent to tRNA gene	
GI16	6077090	6109421	32332	Adjacent to tRNA gene	
GI17	6493076	6508978	15903	Adjacent to tRNA gene	
GI18	7763756	7767905	4150	Containing mobile element	
GI19	9048513	9050527	2015	Containing mobile element	

RiPP, ribosomally synthesized and posttranslationally modified peptides. The GIs that were successfully deleted in *M. xanthus* DK1622 are underlined.

16, a suitable carbon source, and the fed-batch process, the yield of epothilone D in *M. xanthus* increased 140-fold from an initial titer of 0.16 mg/L [188] to 85 mg/L after 22 days of fed-batch cultivation [189]. In addition, the enhancement of epothilone gene transcription by tandem P_{epo} promoters [178] or CRISPR-dCas9 activation [160,161] significantly boosted epothilone production. Thus, there is good reason to believe that the epothilone yield in the heterologous host *M. xanthus* will be significantly enhanced by optimizing culture conditions, combining different approaches, and modifying the producer strain comprehensively.

6. Construction of myxobacterial chassis

Due to the lack of genetic manipulation techniques in most myxobacteria, heterologous expression of smBGCs is an efficient means of exploiting the biosynthetic potentials of myxobacteria, including functional elucidation of cryptic gene clusters and optimization of product yields [190]. However, a high yield is typically not guaranteed when a biosynthetic gene cluster is isolated and transferred to a heterologous host. Different chassis of cyanobacteria [191], actinobacteria [192], filamentous fungi [193], and yeast [194,195] have been successfully constructed and utilized for the heterologous expression of natural active products. However, their performance in expressing smBGCs from myxobacteria was typically poor [196]. In order to achieve efficient heterologous expression and improve product supply for further exploitation of smBGCs from myxobacteria, it is highly desirable to establish an efficient and versatile microbial production platform. Considering issues such as promoter recognition, precursor supply, and self-resistance, we conclude that phylogenetic relatedness is crucial for successful heterologous expression. *M. xanthus* DK1622 is a preferred heterologous host of secondary metabolites from myxobacteria not only due to its phylogenetically close relationship to the native producers but also some advantageous features. The DK1622 strain grows dispersedly in a liquid medium with a shorter generation time (3–4 h) compared to many other myxobacteria, as well as available fermentation knowledge [92]. As a promising producer of natural products, *M. xanthus* DK1622 is characterized by the availability of common biosynthetic precursors [197] and broad spectrum phosphopantetheinyl transferases required for posttranslational activation of PKS/NRPS megasynthetases [186]. Additionally, the genetic manipulation methods developed for myxobacteria are mostly applicable in *M. xanthus* DK1622, making it easier to redesign and renovate the strain as an ideal chassis host [198].

M. xanthus DK1622 is frequently used to heterologously express exogenous smBGCs [7]. The 9.14-Mb genome of DK1622 contains numerous secondary metabolic genes, regulatory genes, and transfer elements,

all of which contribute to the intricate regulatory network [8,121]. This, however, is not conducive to the construction of stable, high yield engineering strains and can easily result in an unstable and unstable strain state [199–201]. Screening the *M. xanthus* DK1622 genome revealed at least 24 smBGCs (Table 4), which are primarily clustered in two regions (4.4–5.8 Mb and 1.5–3.5 Mb), but only nine gene clusters of their products have been identified [7]. The characterized and unidentified secondary metabolites are predominantly PKS/NRPS compounds, which may compete for precursors and energy with the synthesis of the target product. Therefore, the endogenous smBGCs should be deleted to simplify the autochthonous metabolic background and the complexity of the chromosomes. For instance, the myxochromides A gene cluster, one of the most abundant compound families produced by *M. xanthus* DK1622 under standard cultivation conditions, was deleted by homologous recombination and replaced with a tetracycline resistance gene, resulting in the mutant strain Δ*mchA*-tet. The mutant was once used as a host for parallel expression studies involving myxobacterial products such as myxopyronin [92], myxochromides [202], vioprolides [203], and argyrins [89]. Similarly, using the CRISPR-Cas9 system, some smBGCs were deleted in an epothilone-producing *M. xanthus* strain to examine their effects on epothilone production [159]. Regarding mobile elements, IslandPath-DIMBO predicts the existence of 19 genomic islands (GIs), which account for 4.5% of the genome sequence length (Table 4). Moreover, the deletion of 12 of the 19 GIs had no effect on cellular growth (unpublished), but the deletion of multiple GI genes is a time-consuming process.

Genome reduction based on the deletion of non-essential genes, such as competing endogenous smBGCs and mobile elements, will simplify metabolic profiles and increase the genetic stability of chassis cells. Numerous examples indicate that a reduced genome leads to improved growth characteristics (including smaller cell size, reduced cell autolysis, and more dispersed growth), increased electroporation efficiency, and enhanced heterologous protein and secondary metabolite production [204–208]. However, some secondary metabolites that appear to be non-essential may play unknown roles in cell growth, and in some cases, deletion of genomic regions predicted to be non-essential may hinder cell growth [205,209]. Hence, rational construction of genome-reduced myxobacteria chassis with *M. xanthus* DK1622 necessitates an in-depth analysis of dispensable genetic elements and sequential knockout verification of non-essential gene elements, which may be demanding but essential.

In addition to conventional genetic operations such as homologous recombination, site-specific recombination mediated by phage transposase, transposition, and plasmid expression system, new genome editing tools for efficient deletion of large genomic regions and reduction of metabolic background have been developed. Yang et al. develop efficient genome engineering systems for large fragment deletion in *M. xanthus* DK1622 based on the CRISPR-Cas9 system and Cre/loxP-mediated recombination and successfully delete 83-466 kb genomic regions [159,210]. In addition, recently reported endogenous strong constitutive promoters in *M. xanthus* DK1622 provide a crucial synthetic toolkit for efficient gene expression [211]. With the advancement of genome editing tools, a genetically accessible *M. xanthus* DK1622 chassis for the efficient heterologous expression of unexplored secondary metabolites from myxobacteria is highly anticipated.

7. Conclusion and perspectives

The methods of genetic manipulation in myxobacteria and their applications in the exploitation of myxobacterial natural products are summarized in this article. As a rich source of structurally diverse secondary metabolites with intriguing biological activities, myxobacteria exhibit a high potential for the discovery of new drugs with high chemical diversity and uncommon mechanisms of action. The exceptionally large genome sizes and abundance of smBGCs indicate vast untapped natural product resources. Traditional recombination- or transposition-based genetic manipulation, in addition to CRISPR-Cas and Red/ET techniques, enables the operation of myxobacteria and the identification of novel myxobacterial secondary metabolites. As stated previously, the CRISPR-Cas9 system must be optimized prior to its efficient application in *M. xanthus* DK1622, such as the flexible substitution of sgRNA. The NHEJ system is recommended for further development of the CRISPR-Cas system in *M. xanthus* DK1622 because the homologous recombination repair after shearing of Cas9 is dependent on the introduction of homologous arms, which prolongs the cycle of engineering mutant strains. Similarly, Cre/loxP-mediated recombination requires the introduction of two loxP sites in the same orientation; a similar issue exists in the Cre/loxP system. Moreover, similar to the Rec/ET system in *E. coli*, the host-specific Redα/Redβ recombinase has been identified and used for highly efficient genome editing in *S. brevitalea* DSM 7029 [205,212]. Therefore, we anticipate that myxobacteria-specific homologous recombinases will develop more efficient genetic manipulation techniques in *M. xanthus*.

From high-abundance molecules that could be fractionated from grown cultures to massive transcriptionally silent or cryptic smBGCs, we also consider the necessity and maneuverability of constructing myxobacterial chassis for expression and yield optimization of smBGCs. Classic chassis cells such as *E. coli* and *Streptomyces* are incapable of expressing smBGCs from myxobacteria, necessitating the use of myxobacterial chassis cells. As the most well-studied model strain, *M. xanthus* DK1622 is the preferred candidate. The increasingly sophisticated genetic manipulation techniques established in myxobacteria, combined with the flourishing omics tools (genomics, transcriptome, metabolomics, and proteomics), should enable rational modification of *M. xanthus* DK1622 as an ideal chassis. The numerous endogenous smBGCs and GIs are favored as deletion targets for genome reduction. With a simplified host, the optimal transcription and translation of smBGCs are required for the efficient expression of the target product. The establishment of an enriched promoter library has expanded the synthetic biology toolkit available for *M. xanthus* DK1622 [211]. The identification of secondary metabolism regulatory factors, particularly global regulatory factors such as ROK-like proteins [122], will provide potent tools for regulating the transcription of target gene clusters. Considering genetic modification at the translational level, ribosome engineering merits further study in *M. xanthus* DK1622 to activate the silent gene clusters or optimize the production of known products, just as in *Streptomyces* [213]. Although the mechanism is not well understood, some molecular chaperones, such as groEL in *M. xanthus* DK1622 [214], seem to regulate secondary metabolism at a non-transcriptional level, making them important regulatory factors in the myxobacteria chassis.

In the future, natural product mining in myxobacteria is anticipated to be accelerated by the cloning or refactoring of biosynthetic pathways coupled with systematic heterologous expression in optimized chassis cells.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given his role as Editorial Board Member, Dr. Yuezhong Li, had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Linquan Bai.

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