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Engineered production of *iso*-migrastatin in heterologous *Streptomyces* hosts

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ABSTRACT

Glutarimide-containing polyketides such as migrastatin (MGS) are well known for their ability to inhibit tumor cell migration. We have previously shown that MGS is derived from *iso*-migrastatin (*iso*-MGS) via a H_2O-mediated ring-expansion rearrangement. A bacterial artificial chromosome (BAC) library of *Streptomyces platensis* NRRL18993, an *iso*-MGS producer, was constructed. From this library, pBS11001, a BAC clone harboring the intact *iso*-MGS biosynthetic gene cluster, was identified. Mobilization of pBS11001 into five heterologous *Streptomyces* hosts afforded recombinant strains, SB11001, SB11002, SB11003, SB11004, and SB11005, respectively. Under a standard set of media and fermentation conditions, the recombinant strains all produced the same profile of *iso*-MGS as that of *S. platensis* NRRL18993. These findings highlight the strength and flexibility of the BAC-based technology for natural product production and engineering in heterologous *Streptomyces* model hosts.

1. Introduction

'Combinatorial biosynthesis' – in its broadest sense, the generation of novel analogs of natural products by genetic engineering of biosynthetic pathways – complements traditional organic synthetic methods that often bear the burden of high production and environmental costs, particularly as the molecular complexity of target molecules increases. Development of expedient tools for recombinant DNA technologies in *Streptomyces* species and related microorganisms has made possible the application of genetic principles to meet the biotechnological challenge of drug discovery and development in these organisms. Specific, rather than random effects, can often be achieved by this approach, and target metabolites are produced by recombinant organisms amenable to large-scale fermentation.

Successful application of combinatorial biosynthesis requires (i) availability of the gene clusters encoding the synthesis of a given natural product or class thereof, (ii) genetic and biochemical characterization of the selected biosynthetic machinery to an extent sufficient for rational engineering, (iii) expedient genetic systems for in vivo manipulation of genes governing production of the target molecules in their native producers or heterologous hosts, and (iv) production of natural products or their engineered analogs in quantities sufficient for detection, isolation and characterization.[1–4] In particular, successful application of combinatorial biosynthesis requires that the producing microorganism of choice be readily culturable and that the genetics of the host organism is compatible to those of the biosynthetic gene cluster of interest. This realization critically links the strength and flexibility of heterologous model hosts to the potential use of combinatorial biosynthesis strategies on novel natural products found in rare, slow-growing, or difficult to culture microorganisms.[5–7] Notably, only ~1% of soil dwelling microorganisms have been cultivated in the lab and subsequently exploited for drug discovery and development purposes.[8–10] This extraordinarily low, and discovery-limiting number is attributed to media, incubation times and inoculum sizes traditionally used to isolate and expediently culture rare soil bacteria. Advances in culturing techniques have broadened the repertoire of previously unculturable microbes examined for natural products. However, as surely as advances will continue to unveil new natural product scaffolds, the application of combinatorial biosynthesis methods to new biosynthetic gene clusters will require that target gene clusters reside and express in well characterized model organisms that are highly amenable to large-scale fermentation, titer improvement and that do not call for highly specialized culturing methods or equipment.

Among the tools applicable to natural product production and engineering in heterologous hosts are bacterial artificial chromosomes (BACs). BAC vectors can harbor large DNA inserts (up to 600 kb), and BAC-based technology has been widely used in eukaryotic cell biology studies.[11] However, there have been only a few reports of BAC-based strategies to clone large (>40 kb) natural product biosynthetic gene clusters for heterologous

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expression.[12–16] Production of daptomycin, a lipopeptide antibiotic produced by *Streptomyces roseosporus* recently approved for the treatment of bacterial skin infections remains to our knowledge the only successful example. Daptomycin and a small library of engineered analogs were produced in *Streptomyces lividans* TK64 by expression of BAC clones harboring the entire 126-kb *dpt* biosynthetic gene cluster and its engineered progenies.[16]

There is a great need to investigate new hosts (preferably already optimized for high titer production of specific natural products) as producers of natural products for which combinatorial biosynthesis methods have been developed and for which there exists medical significance.[5–7] Although a truly "universal" expression system suitable for all classes of natural products from all natural sources is unrealistic, the development of a suite of *Streptomyces* hosts enabling highly flexible and amplified production of a wide range of natural products from actinomycetes, represents a highly significant endeavor.

We are actively engaged in biosynthetic studies of the glutarimide-containing polyketides *iso*-migrastatin (*iso*-MGS), migrastatin (MGS), and the dorrigocins (DGNs) in *Streptomyces platensis* NRRL18993.[17–20] Members of this class are potent inhibitors of human tumor cell migration [21–25] and thus hold tremendous potential as inhibitors of tumor metastasis; metastasis being integrally dependent upon cell migration processes.[26–28] Genome scanning of *S. platensis* NRRL18993 unveiled at least two glutarimide-containing polyketide biosynthetic loci, named the *mgs* (also known as *DORR*) and *088* clusters, respectively.[29] However, inactivation of the *mgs* locus alone was sufficient to abolish the production of *iso*-MGS, MGS, and DGNs, suggesting that these metabolites share the same biosynthetic machinery.[17] Since these early efforts, we have subsequently shown that neither MGS nor the DGNs are bona fide natural products.[17] Rather, they are shunt metabolites of *iso*-MGS, derived from a H_2O-mediated ring-expansion and ring-opening rearrangement of *iso*-MGS (Fig. 1).[18,19] We also reported that *iso*-MGS can undergo a concerted [3,3]-sigmatropic rearrangement under neat heating conditions to afford MGS regio- and enantiospecifically.[19]

Predicated on the importance of *iso*-MGS to the glutarimide-containing polyketide class of natural products, enhanced production methods for *iso*-MGS are likely to make related compounds of medicinal interest more readily available. Moreover, we have also begun to develop combinatorial biosynthetic methods applicable to the glutarimide-containing polyketides. In developing a suite of heterologous hosts capable of producing *iso*-MGS and related analogs (engineered or naturally derived) we report here (i) strategy for, and construction of, BAC clones capable of harboring large biosynthetic gene clusters (up to 200 kb), (ii) isolation of BAC clones from *S. platensis* NRRL18993 that harbor the *iso*-MGS gene cluster, (iii) mobilization of the BAC clones into five heterologous *Streptomyces* hosts, and (iv) production of *iso*-MGS in the resultant recombinant strains. These results validate the BAC-based technology for expressing large biosynthetic gene clusters for natural product production and engineering in heterologous hosts.

2. Results

2.1. BAC vector design for cloning of large biosynthetic gene clusters for expression in *Streptomyces* hosts

pStreptoBAC V, the BAC vector developed for cloning and expression of daptomycin in heterologous *Streptomyces* species,[16] was adopted for this work. pStreptoBAC V features a set of carefully engineered characteristics, including (i) the origin of replication derived from pBACCe3.6 which allows maintenance of the BAC DNA in *Escherichia coli*, (ii) the *Streptomyces* phage *ΦC31* integrase responsible for site-specific integration into the *att* site of the chromosome, thereby stably maintaining the BAC in the heterologous *Streptomyces* host, (iii) the *oriT* element to facilitate efficient transfer between *E. coli* and *Streptomyces* by conjugation, (iv) the

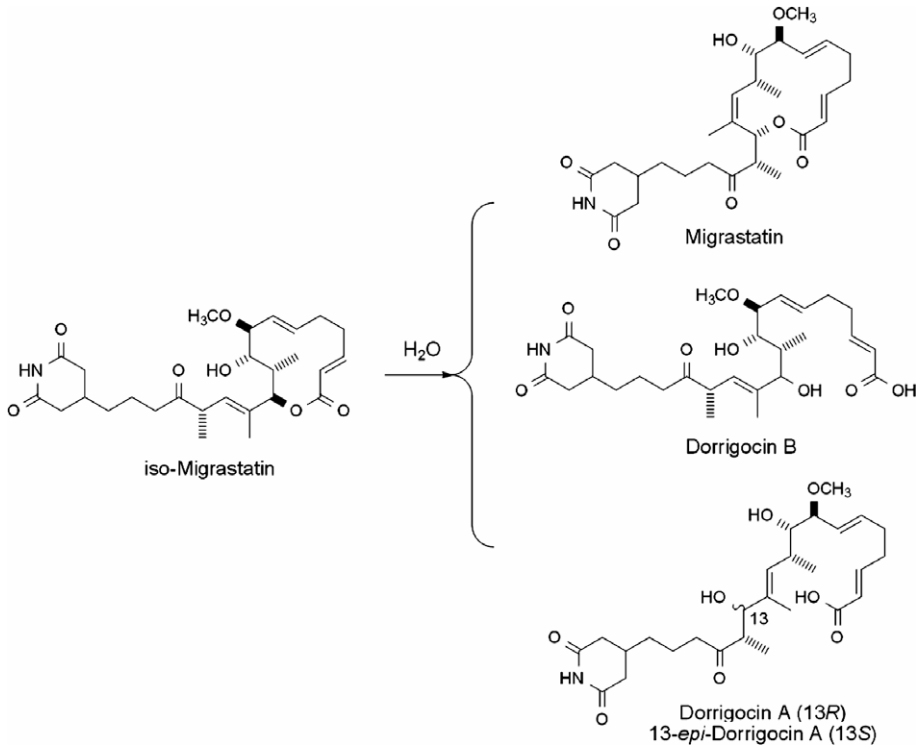

Figure 1. Glutarimide-containing polyketides isolated from *S. platensis* NRRL18993: *iso*-migrastatin and its H_2O-mediated ring-expansion and ring-opening rearrangements to migrastatin and dorrigocins.

aac3(IV) apramycin resistance gene for use of apramycin as a selectable marker in both *E. coli* and *Streptomyces*, and (v) the BamHI sites for convenient cloning of the target natural product biosynthetic gene cluster in BAC library construction (Fig. 2A).[16]

The pStreptoBAC V vector is also characterized by the replication region from the high-copy-number plasmid pUC19 which resides between the two cloning sites. This was designed to enable a high-copy-number of the pStreptoBAC V vector to be maintained thus facilitating its preparation. For BAC library construction the pUC19 replication region in pStreptoBAC V was removed via BamHI digestion and the remaining vector ligated with large DNA fragments from *S. platensis*. As a result, BAC clones composing the initial library remained single copy in *E. coli*.[16]

2.2. Construction of the *S. platensis* NRRL18993 BAC library and isolation of the BAC clone pBS11001 containing the intact *iso*-MGS gene cluster

To construct the BAC library, *S. platensis* NRRL18993 chromosomal DNA was prepared in agarose plugs and partially digested with BamHI. The resulting DNA fragments were size-fractionated by two rounds of pulse field gel electrophoresis (PFGE). DNA fragments ranging in size from 100 to 250 kb were ligated with BamHI-digested pStreptoBAC V vector. Ligation mixtures were then transformed into *E. coli* DH10B competent cells by electroporation. Approximately 1000 transformants were obtained per 20 ng of DNA. The average insert size was 75 kb as determined by PFGE analysis of HindIII digests from 18 randomly selected clones (Fig. 3).

Genome scanning of *S. platensis* NRRL18993 has previously unveiled at least two loci, *mgs* and *088*, whose deduced biosynthetic machineries were consistent with glutarimide-containing polyketide biosynthesis.[17,29] However, gene inactivation suggested that the *mgs* locus was the one responsible for *iso*-MGS biosynthesis.[17] To isolate BAC clones that harbor the intact *mgs* cluster, the *S. platensis* NRRL18993 library (2400 clones) was first screened by hybridization using an upstream gene (*mgsA*) of the sequenced *mgs* locus as a probe; 17 positive clones were identified in this manner. Positive clones were then screened by PCR using primers from a downstream gene (*mgsK*) of the sequenced *mgs* locus. From this screen was identified one positive clone, pBS11001, containing a 65-kb insert, end-sequencing of which confirmed that it contains the complete *mgs* gene cluster (Fig. 2B).

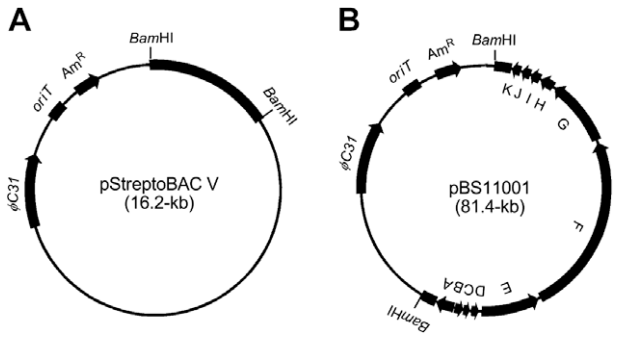

Figure 2. BAC-based technology for natural product production and engineering in heterologous *Streptomyces* hosts: (A) the pStreptoBAC V vector, featuring two BamHI sites, the apramycin resistance marker (Am^R), *oriT*, and *ΦC31* and (B) the pBS11001 clone with 65-kb DNA insert from *S. platensis* NRRL18993 that harbors the intact *mgs* biosynthetic gene cluster.

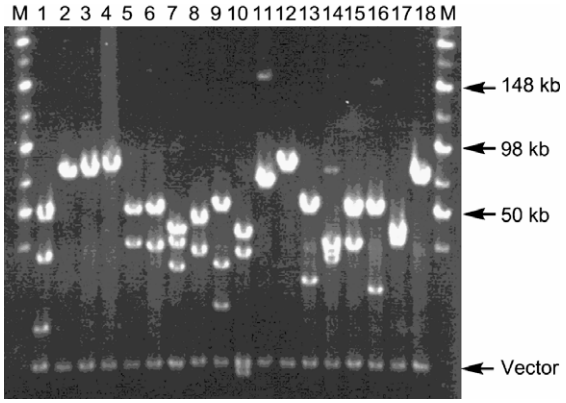

Figure 3. Analysis of BAC clones of *S. platensis* NRRL18992 library. Eighteen randomly picked BAC clones were digested with HindIII and the digested DNAs were analyzed by pulsed field gel electrophoresis (PFGE). Lanes 1–18, HindIII-digested BAC DNAs; M, DNA marker, with relevant sizes noted on right.

2.3. Mobilization of pBS11001 into heterologous *Streptomyces* hosts

pBS11001 was mobilized into *Streptomyces albus* J1074, *S. lividans* K4-114, *Streptomyces coelicolor* M512, *Streptomyces avermitilis* SUKA4, and *S. avermitilis* SUKA5 via conjugation. Apramycin resistant exconjugants were selected, isolated, purified, and named SB11001 [*S. albus* J1074(pBS11001)], SB11002 [*S. lividans* K4-114(pBS11001)], SB11003 [*S. coelicolor* M512(pBS11001)], SB11004 [*S. avermitilis* SUKA4(pBS11001)], and SB11005 [*S. avermitilis* SUKA5(pBS11001)], respectively. Integrations of pBS11001 into the chromosomes of the heterologous *Streptomyces* hosts were verified by PCR using primers *mgsK*-F and *mgsK*-R, designed according to the *mgsK* sequence. The desired ~800-bp PCR products were obtained from all recombinant strains, indicating the correct integration of pBS11001 into the chromosome of each heterologous host.

2.4. Production of *iso*-MGS in heterologous *Streptomyces* hosts

Before exerting extensive efforts to optimize *iso*-MGS titers for each host, the five recombinant strains, with the *S. platensis* NRRL18993 as a control, were subjected to a standard set of media and fermentation conditions to examine if *iso*-MGS was produced in each of the heterologous *Streptomyces* hosts. Thus, each of the five recombinant strains SB11001, SB11002, SB11003, SB11004, or SB11005 was grown in B2 or R2YE medium, respectively. Incubations were conducted for 4–5 days in the presence of 5% amberlite XAD-16 resin, after which time secondary metabolites were collected from culture broth via resin isolation and extraction from resins with ethanol.[17–20] Crude extracts were analyzed by HPLC, and *iso*-MGS was identified on the basis of retention time, UV–vis spectroscopy, co-injections with authentic *iso*-MGS and LC–MS analysis. As shown in Figure 4, all recombinant strains carrying pBS11001 produced *iso*-MGS as the major product that possesses an identical retention time, displays an identical UV–vis spectrum, and yielded identical $[M+H]^+$ and $[M+Na]^+$ ions upon LC–MS analysis to that of an authentic standard.[17–19] Finally, to unambiguously confirm the identity of *iso*-MGS produced in the heterologous hosts, the major product was isolated from SB11001 for NMR analysis; both ^1H and ^{13}C NMR spectra were identical to those of authentic *iso*-MGS.[17–19] These data, taken together, confirm the production of *iso*-MGS by expression of the *mgs* gene cluster in heterologous hosts.

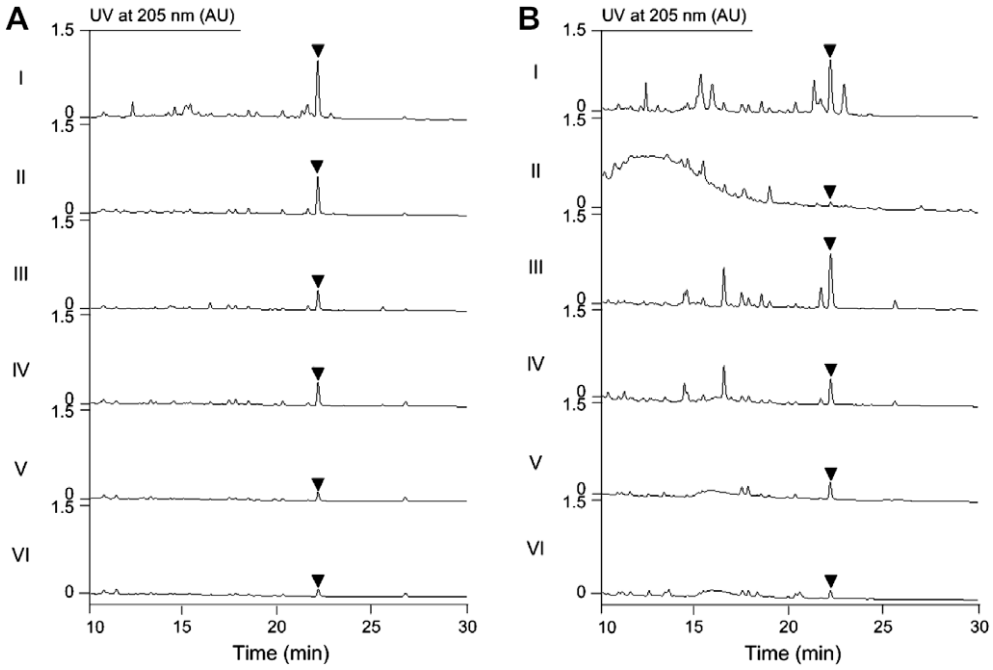

Figure 4. HPLC analysis of extracts from recombinant strains SB11001 (II), SB11002 (III), SB11003 (IV), SB11004 (V), SB11005 (VI), with *S. platensis* NRRL18993 as a control (I), cultured in (A) B2 or (B) R2YE medium. ▼, *iso*-MGS. Normalization of *iso*-MGS signal intensity between panel A and B analyses required that five times as much R2YE-derived extract be injected compared to the corresponding B2-derived extract (see Section 5.5).

Table 1
iso-MGS production titers in wild-type and recombinant strains.

Strains	Titer (mg/L)	
	B2 medium	R2YE medium
S. platensis NRRl18993	58 ± 8	17 ± 1
S. albus J1074(pBS11001) (SB11001)	46 ± 4	0.6 ± 0.3
S. lividans K4-114(pBS11001) (SB11002)	25 ± 2	15 ± 1
S. coelicolor M512(pBS11001) (SB11003)	23 ± 4	5.3 ± 1
S. avermitilis SUKA4(pBS11001) (SB11004)	2.6 ± 0.6	3.4 ± 0.8
S. avermitilis SUKA5(pBS11001) (SB11005)	4.2 ± 0.4	1.3 ± 0.4

The titers of *iso*-MGS from the recombinant strains were then determined by HPLC analysis with the *S. platensis* NRRL18993 as a control,[17–19] and these data are shown in Table 1. The yields of *iso*-MGS from the heterologous hosts are lower than those noted for *S. platensis* NRRL18993 under the conditions examined, and yields in B2 medium universally surpass those obtained using R2YE medium. This is not surprising given the fact that the B2 medium and the fermentation conditions examined were optimized previously for *iso*-MGS production in *S. platensis* NRRL18993.[17–19]

3. Discussion

More than 70% of antibiotics and anticancer drugs in use today are natural products or bear structures inspired by natural products.[30,31] Thus, despite a move away from natural products during the 1990s, these compounds remain the best source of leads for new drug discovery efforts. Most antibiotics are isolated from microorganisms and are the products of secondary metabolite machinery. Despite their wealth of structure diversity and creative occupancy of chemical space which has proven very important in the identification of new drug chemotypes, natural product titers are often limited by the poor growth of the original microbial producer. Thus, securing significant quantities of secondary metabolites from their native producers can often be challenging, if not rate-limiting, to the drug development process. Moreover, it has been estimated that greater than 99% of the environmental microbes responsible for natural product biosynthesis are not culturable in the laboratory setting.[8–10] However, difficulties with microbial cultivation and natural product titers can be circumvented via the use of heterologous hosts.[5–7] Such hosts are able to house a given biosynthetic gene cluster in a readily cultivatable system that often possesses a genome more amenable to improved natural product titers and is, ideally, permissive of a wider array of genetic modifications to the integrated biosynthetic gene cluster. The latter consideration is particularly important from a perspective of combinatorial biosynthesis.

It is not uncommon to use multiple-plasmids harboring different parts of one gene cluster[32] or to incorporate multiple-steps to clone a gene cluster from different cosmid clones into one plasmid to achieve heterologous expression.[33,34] Multi-step strategies were popularized following the development of Red/ET recombinant technologies but continue to involve multiple manipulations.[35,36] Alternatively, BAC plasmids, with the ability to harbor large DNA, make possible one step cloning of large biosynthetic gene clusters.[11–16] BACs capable of shuttling between *E. coli* and *Streptomyces* are particularly attractive, providing a means to conveniently transfer large natural product biosynthetic gene clusters from *E. coli* to *Streptomyces*.[12,16] Despite the fact that such an attempt was first reported almost a decade ago,[12–16] examples of BAC-based production of natural products in heterologous *Streptomyces* hosts remains very limited. Underlying the significance of findings reported here, to our knowledge, only the daptomycin gene cluster has been successfully expressed in the heterologous *Streptomyces* host using the BAC-based technology.[16]

An ideal heterologous host should grow quickly, be genetically amenable to manipulation, provide all necessary precursors and have little or no significant capacity for competitive endogenous secondary metabolite production.[5–7] Moreover, there must be in place a facile means by which to integrate and ensure functional expression of large biosynthetic clusters within the host. *S. coelicolor* and *S. avermitilis* are routinely used as heterologous

Streptomyces hosts in large part because of the extent to which their genomes are understood including genome sequencing.[37,38] Although the genomics of *S. albus* is not as well defined as for *S. coelicolor* or *S. avermitilis*, it also has been widely used because of its genetic amenability and rapid rate of growth.[5–7] In this study, we used *S. coelicolor* M512 whose production of red pigment undecycloprodigiosin (Red) and actinorhodin (Act) has been abolished by inactivation of the *afsR* regulatory gene.[39] *S. lividans* K4-114 whose entire *act* gene cluster has been deleted,[40] and *S. albus* J1074, a fast-growing strain known as an empirically excellent heterologous hosts for natural product production,[6] also were selected as hosts for *iso*-MGS production. The entire genome of *S. avermitilis* ATCC31267 has been sequenced,[38] and two loci containing putative biosynthetic gene clusters were deleted from its chromosome to generate *S. avermitilis* SUKA4 and SUKA5, which are unable to produce the endogenous panel of metabolites including avermectin, oligomycin, polyenes, carotenoids, melanins, and two nonribosomal peptides. These *S. avermitilis* strains also were used as hosts allowing one to assess the impact of endogenous secondary metabolite pathways upon *iso*-MGS production. pBS11001 harboring the intact *mgs* cluster was integrated into the chromosome of all hosts. The resultant recombinant strains SB11001, SB11002, SB11003, SB11004, and SB11005 were all found to produce *iso*-MGS under the conditions originally optimized for the native producer *S. platensis* NRRL18993 (Fig. 4 and Table 1).

Pending fermentation optimization for each recombinant strain, the *iso*-MGS yield from heterologous hosts is, in some cases, on par with the native producer *S. platensis* NRRL18993. Conversely, production of *iso*-MGS in some host and medium combination is much lower than that with the native producer. While this may be attributed to a number of factors, including promoters, precursor availability, and competing biosynthetic pathways,[5–7] it should be pointed out that the choice of media and fermentation conditions examined are biased towards the native producer *S. platensis* NRRL18993.[17–19] Fermentation optimization would be the next logical step to improve *iso*-MGS titers in the recombinant strains.

Important to note is the impact of fermentation medium upon product profiles for all hosts evaluated. Both the native and recombinant strains for *iso*-MGS production produce what is clearly a more complex product profile following fermentation in R2YE than is the case for growth in B2 (Fig. 4). This is most evident for *iso*-MGS production in SB11001 where the titer of 46 mg/L decreased to less than 1 mg/L in going from B2 to R2YE medium (Table 1). Any of the reasons noted above might explain this change although examination of Figure 4 makes clear that *iso*-MGS production in SB11001 in B2 is significantly cleaner than in R2YE. Within the margins of standard error, this is the case for all recombinant strains evaluated strongly suggesting that competing metabolic pathways detract from *iso*-MGS production in a manner partly driven by the R2YE medium. Nonetheless, we have demonstrated that BAC-based technology is highly practical and can be used as a general technology for producing natural products in high yields upon expression of the target gene cluster in selected model heterologous hosts. To achieve the highest titer of the target natural product in the recombinant strains, however, may require additional optimization of medium and fermentation conditions.

A practical technology for reliable production of natural products in user-friendly hosts provides tremendous opportunity to access natural products that cannot be accessed by other traditional means and allows the rapid correlation of genomics and bioinformatics information to specific natural products. Tools readily available for these model heterologous hosts can now be fully exploited for natural product production and engineering. The rewards of such efforts include a hastened ability to dissect biosynthetic pathways, as well as, the ability to enhance natural product titers and to engineer new analogs. Particularly appealing is the idea that a biosynthetic cluster (and associated secondary metabolite) whose importance might be marginalized by the genetics of its native producer can be made significantly more useful by simple transfer to a better host.

4. Conclusions

BAC-based technology was used to express the *mgs* gene cluster from *S. platensis* NRRL18993 in heterologous *Streptomyces* hosts. pBS11001, a BAC clone containing the intact *mgs* gene cluster, was obtained from the *S. platensis* NRRL18993 BAC library and transferred into five heterologous *Streptomyces* hosts. *iso*-MGS production was realized in all five resultant recombinant strains, highlighting BAC-based heterologous expression as a practical technology for natural product production and the broad exploitation of combinatorial biosynthesis methods for production of new natural product analogs not readily attained in their native producers or via conventional synthetic strategies.

5. Experimentals

5.1. Bacterial strains, plasmids and culture conditions

E. coli strains DH10B[41] and ET12567/pUZ8002[42] were grown on solid or in liquid LB medium[41] at 37 °C. *S. platensis* NRRL18993[17–19] was cultured in TSB (Difco, Lawrence, KS) for 2 days at 28 °C for isolation of total DNA.[42] *S. platensis* NRRL18993, *S. albus* J1074,[6] *S. lividans* K4-114,[40] and *S. coelicolor* M512[39] were grown on ISP4 (Difco) medium for sporulation, while YMS medium[43] was used for sporulation of *S. avermitilis* SUKA4 and SUKA5 strains (gift of Haruo Ikeda, Kitasato University, Japan). Antibiotics were added when necessary at the following concentrations: kanamycin, 50 μg/mL; apramycin, 25 μg/mL; ampicillin, 50 μg/mL.[41,42]

5.2. Genetic manipulations

DNA manipulation was carried using standard methods.[41,42] Genomic DNA of *S. platensis* NRRL18993 was isolated according to a general protocol.[42] Unless specifically noted, restriction enzymes and other molecular biology reagents were purchased from Invitrogen and used as per manufacturer instructions. PCR amplification was carried out on a GeneAmp® PCR system 2400 (Applied Biosystems, Foster City, CA) with *Takara LA Taq*™ (Takara Bio USA, Madison, WI) with the supplied GC buffer II. Southern blotting was performed according to the standard protocols[41] using the DIG-system (Roche, Palo Alto, CA). Appropriate DNA fragments were labeled with DIG-labelled dUTP and used as probes. BAC DNA was introduced into heterologous hosts by conjugation following literature methods.[42]

5.3. Construction of *S. platensis* NRRL18993 genomic BAC library and library screening

S. platensis NRRL18993 was cultured in TSBY medium[42] containing 0.5% glycine for 2 days. The cells were washed twice with water and resuspended in 2 volumes of suspension buffer (10 mM Tris, 100 mM EDTA, pH 8.0). The above mixture was mixed 1:1 with 1.2% certified™ Low Melt Agarose (Bio-Rad, Hercules, CA) and put into the plug module. After solidification, the plugs were immersed in lysozyme buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 0.5% sodium lauryl sarcosine, 1 mg/mL lysozyme) and placed at 37 °C for 12 h. The buffer was then replaced by proteinase buffer (10 mM Tris, pH 8.0, 400 mM EDTA, 1% sodium lauryl sarcosine, 0.1%, 1 mg/mL proteinase K). The tube containing the plugs was placed at 50 °C for 48 h with one proteinase buffer change at 24 h. After being washed 5 times with TE buffer, the plugs were used for

partial digestion. Partial digestion of genomic DNA in plugs (10) was performed in digestion buffer (20 U BamHI, 50 mM Tris, 10 mM MgCl, 100 mM NaCl, 1 mM DTT, 0.01% BSA) for 20 min at 37 °C on ice. The DNA fragments in the plug were separated by PFGE twice on 1% certified™ Low Melt Agarose (Bio-Rad) as follows: 1st PFGE, 4 V/cm with a pulse time of 1–50 s for 24 h; cutting out the agarose band containing DNA fragments ranging between 100–250 kb; subjecting the resultant DNA fragments to 2nd PFGE, 4 V/cm with a pulse time of 3–8 s for 20 h. After PFGE, the low melt agarose containing DNA fragments between 100 and 250 kb were digested with GLase (Epicentre, Madison, WI) and the solution was ligated with prepared BAC vector directly by using Fast-Link DNA ligase (Epicentre, Madison, WI). Ligation was performed at 16 °C overnight. The ligation mixture was transformed into MegeX DH10B™ Electrocomp™ *E. coli* (Invitrogen, Madison, WI) by electroporation (1.5 kV/mm) using an *E. coli* Pulser (Bio-Rad). BAC DNA was extracted from randomly selected BAC clones and HindIII-digested DNAs were run on PFGE (5–12 s in 14 h, 6 V/cm) to check the insert size. The BAC clones were picked from the plate and placed into freezing medium in 96-well microplates, cultured overnight and then stored at −80 °C. The *S. platensis* NRRL18993 BAC library clones were transferred onto Hybond-N+ membranes (Amersham Pharmacia, Piscataway, NJ) and fixed for hybridization.[41] The membranes were hybridized with the *mgsA* probe, which was amplified with primers *mgsA*-F(5′-AGCGAAG GAGCGGTCGGAGGG-3′) and *mgsA*-R (5′-GTCAGGTGGCGGACGAG GAGA-3′). The positive clones were then screened by PCR with primers *mgsK*-F (5′-CTCGACCCGCATCTCAGCTCCGA-3′) and *mgsK*-R (5′-CGTCCAGCTTTCCGGGGTTCTCG-3′) using the following conditions: 94 °C for 2 min; 35 cycles each of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; 72 °C for 7 min.

5.4. Fermentation of the recombinant strains and *S. platensis* NRRL18993

Fermentations of the recombinant strains, with *S. platensis* NRRL18993 as a control, were carried out by following the published procedures.[17–19] Two different media were used for host fermentations. B2 medium has been previously used for *iso*-MGS and related glutarimide productions.[17–20] R2YE medium[42] also was evaluated as a fermentation medium; both B2 and R2YE media contained 5% Amberlite® XAD-16 resin (Sigma) as a critical element permitting facile metabolite isolation following fermentation.[17–19] Spore suspensions (50 μL) of *S. platensis* NRRL18993 and the recombinant strains SB11001, SB11002, SB11003, SB11004, and SB11005 were added to 50 mL of seed medium in 250-mL flasks, respectively. The seed cultures were incubated on a rotary shaker at 250 rpm and 28 °C for 30–48 h until the cells grew to log phase. The resulting seed culture (2.5 mL, i.e., 5% of the production medium) was then added to 50 mL of production media in 250-mL flasks, which were incubated on a rotary shaker at 250 rpm and 28 °C for 4–5 days for each strain, respectively.

5.5. Isolation and HPLC and LC–MS analysis of *iso*-MGS from the recombinant strains and *S. platensis* NRRL18993

Isolation of *iso*-MGS from the recombinant and *S. platensis* fermentations followed the methods previously described.[17–19] The significantly more complex metabolite profiles of R2YE-derived extracts relative to their B2 counterparts required that more of each R2YE-derived extract be injected relative to the corresponding B2-derived extract. Resin-derived extracts from 50 mL of cultures in B2 or R2YE medium were dissolved in either 10 or 1 mL of EtOH, respectively. HPLC injection volumes of the B2- and R2YE-derived extracts were 20 and 10 μL, respectively. Analytical HPLC was carried out on a Varian system equipped with in-line Prostar 330 detector (Woburn, MA). The mobile phase was comprised of buffer A (15% CH_3CN in H_2O containing 0.1% HOAc) and buffer B (80% CH_3CN in H_2O containing 0.1% HOAc). LC–MS was carried out on an Agilent 1100 HPLC–MSD SL quadrupole mass spectrometer equipped with both orthogonal pneumatically assisted electrospray and atmospheric pressure chemical ionization sources (Santa Clara, CA). Analytical HPLC and LC–MS was conducted using a Microsorb-MV C18 column (250 × 4.6 mm, 5 μm) (Varian Inc. Palo Alto, CA) eluted with a linear gradient of 100% buffer A and 0% buffer B to 20% buffer A and 80% buffer B over 20 min, followed by 10 min at 20% buffer A and 80% buffer B at a flow rate of 1.0 mL/min with UV detection at 205 nm. LC–MS analysis of the major product in the recombinant strains, with retention time identical to *iso*-MGS, afforded the $[M+H]^+$, $[M+H_2O]^+$, and $[M+Na]^+$ ions at *m/z* of 490.4, 507.4, and 512.4, respectively. ^1H and ^{13}C NMR data were acquired on a VARIAN Inova-500 (500 MHz) spectrometer. The sample was dissolved in $CDCl_3$ with TMS as an internal standard.

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