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Engineering *Salinispora tropica* for heterologous expression of natural product biosynthetic gene clusters

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Abstract

The marine actinomycete genus *Salinispora* is a remarkably prolific source of structurally diverse and biologically active secondary metabolites. Herein, we select the model organism *Salinispora tropica* CNB-440 for development as a heterologous host for the expression of biosynthetic gene clusters (BGCs) to complement well-established *Streptomyces* host strains. In order to create an integratable host with a clean background of secondary metabolism, we replaced three genes (*salA–C*) essential for salinosporamide biosynthesis with a cassette containing the *Streptomyces coelicolor* Φ C31 phage attachment site *attB* to generate the mutant *S. tropica* CNB-4401 via double-crossover recombination. This mutagenesis not only knocks-in the attachment site *attB* in the genome of *S. tropica* CNB-440 but also abolishes production of the salinosporamides, thereby simplifying the strain's chemical background. We validated this new heterologous host with the successful integration and expression of the thiolactomycin BGC that we recently identified in several *S. pacifica* strains. When compared to the extensively engineered superhost *S. coelicolor* M1152, the production of thiolactomycins from *S. tropica* CNB-4401 was approximately 3-fold higher. To the best of our knowledge, this is the first example of using a marine actinomycete as a heterologous host for natural product BGC expression. The established heterologous host may provide a useful platform to accelerate the discovery of novel natural products and engineer biosynthetic pathways.

Keywords

Heterologous expression; *Salinispora*; Natural products; Genetic engineering

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Introduction

Microbially produced natural products are of paramount importance in human medicine. Not only are the majority of antimicrobial and anticancer drugs derived directly or inspired by natural products, many other branches of medicine, such as immunology, neurology, and cardiology, have similarly benefited from them (Newman and Cragg 2016). Bacteria in the order *Actinomycetales*, commonly called actinomycetes, are the undisputed leading source for natural product discovery, accounting for approximately 75% of the microbial natural products used in human therapy (Berdy 2005). Among the actinomycetes, the genus *Streptomyces* has been the major source of bioactive compounds discovered to date and has thus become the model organism for the study of natural product biosynthesis, pathway engineering, and genome mining (Baltz 2016; Chater 2016; Horinouchi 2007). Recently, however, common soil-derived *Streptomyces* bacteria have fallen out of favor as a resource for drug discovery due in part to the common re-discovery of well-known compounds. This has led to a search for other Actinobacteria and the exploration of actinomycetes from poorly exploited environments such as the ocean (Jensen et al. 2005; Schorn et al. 2016).

The marine actinomycete genus *Salinispora* was initially isolated from marine sediments and subsequently described as the first obligate marine actinomycete genus (Jensen et al. 2015). Since then, *Salinispora* species have displayed the ability to produce a diverse range of secondary metabolites, including salinosporamide A, which is currently in clinical trials as an anticancer agent (Gulder and Moore 2010). To date, 25 classes of compounds have been discovered from this genus, with 16 representing new carbon skeletons, correlating to virtually all known biosynthetic classes (Jensen et al. 2015). Recent genome sequencing efforts have revealed that only a small fraction of the natural product biosynthetic potential of *Salinispora* species has been identified (Amos et al. 2017; Letzel et al. 2017; Ziemert et al. 2014). In a recent study, the genomes of 75 *Salinispora* strains were queried for nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) biosynthetic gene clusters (BGCs), revealing 124 distinct biosynthetic pathways predicted to encode 124 distinct natural products (Ziemert et al. 2014). This provides an unprecedented opportunity to explore the biosynthetic potential of *Salinispora* to discover new compounds.

Heterologous expression of whole BGCs has become a routine approach not only for the discovery of metabolites from “orphan” or “cryptic” gene clusters (Ren et al. 2017; Rutledge and Challis 2015), but also to probe biosynthetic logic involved in natural product biosynthesis (Gomez-Escribano and Bibb 2014; Ongley et al. 2013). This approach relies on two factors: first, the ability to clone BGCs into a suitable expression vector, and second, the availability of a compatible heterologous host. Recently, several whole-pathway direct cloning approaches, such as full-length RecE-mediated recombination in *E. coli* (Fu et al. 2012), transformation-associated recombination (TAR) in *Saccharomyces cerevisiae* (Kim et al. 2010; Yamanaka et al. 2014), and Cas9-Assisted *in vitro* assembly method (Jiang et al. 2015), have been developed, and these have greatly accelerated the BGC isolation procedure. On the other hand, several *Streptomyces* strains have been established for heterologous expression of natural product biosynthesis genes (Gomez-Escribano and Bibb 2011; Komatsu et al. 2013; Komatsu et al. 2010; Zhang et al. 2017b). However, empirically, we have found that some *Salinispora* gene clusters, such as those encoding the

salinosporamide (Eustaquio et al. 2009), lymphostin (Miyanaga et al. 2011), and lomaiviticin pathways (Kersten et al. 2013), are active in *S. tropica* strains but inactive when introduced to a *Streptomyces* heterologous host such as *S. coelicolor* or *S. lividans*. The expression of a BGC is a complicated cellular process that is highly dependent on the genetics and biochemistry of the producing organism (Baltz 2010). We believe that the ability to easily test the expression of BGCs across various host organisms is a valuable asset to maximize the likelihood of achieving successful heterologous production of natural product small molecules (Zhang et al. 2017a). Therefore, establishing a biosynthetically versatile organism such as *Salinispora* as a heterologous host may facilitate the expression of additional BGCs for future discovery and engineering efforts.

S. tropica CNB-440 was the first whole genome-sequenced marine actinomycete and has been used as a model organism to investigate secondary metabolites (Udwary et al. 2007). So far, eight classes of compounds have been discovered from this strain alone, including the beta-lactone proteasome inhibitor salinosporamide A (Eustaquio et al. 2009) and the polyketide cytotoxin lomaiviticin A (Kersten et al. 2013). Here, we report the engineering of *S. tropica* CNB-440 as a heterologous host by introducing the phage Φ C31 attachment site *attB* into the genome for integratable expression and simultaneously abolishing background salinisporamide production. The new *Salinispora* host is directly compatible with all previously established genetic toolkits leveraging the phage Φ C31 integrase for *Streptomyces* expression (Tang et al. 2015; Yamanaka et al. 2014). We validated this new heterologous platform with the successful expression of the thiolactomycin BGC recently identified in several *S. pacifica* strains but absent in *S. tropica* (Tang et al. 2015). To the best of our knowledge, this is the first showcase of the engineering and use of a marine actinomycete as a heterologous host for natural product pathway expression.

Materials and methods

Bacterial strains, plasmids, and culture conditions.

Chemicals and microbiological and molecular biological agents were purchased from standard commercial sources. Strains and plasmids used in this study are summarized in Table 1. *Salinispora tropica* CNB-440 (ATCC BAA-916 and DSM 44818) and its respective derivatives were maintained and grown on A1 agar (10 g soluble starch, 4 g yeast extract, 2 g peptone, 16 g agar, 1 liter natural or artificial seawater) and/or A1 liquid medium (10 g soluble starch, 4 g yeast extract, 2 g peptone, 1 liter natural or artificial seawater). *Escherichia coli* strains were cultivated in LB medium (components purchased from BD Biosciences or Fisher Scientific) supplemented with the appropriate antibiotics. DNA isolation and manipulations were carried out according to standard methods for *E. coli* and *Salinispora*.

Construction of a gene disruption cassette containing the *attB* attachment site.

A 500 bp fragment containing the Φ C31 phage attachment site *attB* was amplified from the gDNA of *Streptomyces coelicolor* M1152 (Gomez-Escribano and Bibb 2011) with primers attBreg-F and attBreg-R (Table S1). The gene disruption cassette *acc(3)IV* (Apra^R) and *oriT_{RK2}* were jointly amplified from pIJ773 (Gust et al. 2003) with the primers P1reg-F and

P2reg-R (Table S1). These two PCR products were assembled by an overlap extension PCR reaction using the primers attBreg-F and P2reg-R (Table S1). The final disruption cassette was further cloned into pCR™2.1-TOPO® vector (Fisher Scientific, Hampton, NH, USA) to give pMXT01 and was verified by restriction analysis and sequencing.

Gene replacement on the fosmid BHXS1782 containing the salinosporamide gene cluster.

The disruption cassette containing the Φ C31 phage attachment site *attB*, *acc(3)IV* (Apra^R), and *oriT_{RK2}* were jointly amplified from the 1,961-bp NotI/SpeI fragment of pMXT01 with the primer pair of *salA-C_KO-F/salA-C_KO-R* (Table S1). The PCR products were used to replace the genes *salA-C* on the fosmid BHXS1782 (Eustaquio et al. 2009) in *E. coli* BW25113/pKD20 (Datsenko and Wanner 2000) using the PCR targeting system. The resulting fosmid pMXT04 was confirmed by restriction analysis. Excision of the apramycin resistance cassette was performed in *E. coli* BT340 (Cherepanov and Wackernagel 1995), taking advantage of the FLP recognition sites surrounding the resistance cassette. *E. coli* clones containing the fosmid pMXT10 were screened for apramycin sensitivity and verified by restriction analysis and PCR using the primer pair *SalA-C-test650-F/SalA-C-test650-R* (Table S1). The chloramphenicol resistance marker *cat* on pMXT10 was replaced by the cassette containing *oriT_{RK2}* and *aac(3)IV* (Apra^R), which was amplified from pIJ773 with primers Cat-knockout-F1 and Cat-knockout-R1 (Table S1), to give pMXT11.

Allelic exchange in *Salinispora tropica* CNB-440.

The modified fosmid pMXT11 were introduced into *E. coli* ET12567/pUZ8002 (Paget et al. 1999) by electroporation and then transferred to *S. tropica* CNB-440 by conjugation. Apramycin resistant (Apra^R) exconjugants were identified, indicating a single-crossover allelic exchange in *S. tropica* CNB-440. Single colonies were picked and streaked on A1 agar plates without any antibiotics. Apramycin sensitive colonies were further screened by PCR to obtain the double-crossover allelic exchange mutant *S. tropica* CNB-4401.

Heterologous expression of the thiolactomycin gene cluster.

The plasmid pMXT13 (Tang et al. 2015), containing the thiolactomycin BGC (*tlm*), was transferred into *E. coli* ET12567 and introduced into *S. tropica* CNB-4401 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307 (Flett et al. 1997). 200 μ l overnight culture of *E. coli* was inoculated into 10 mL fresh LB medium plus appropriate antibiotics and grown for 3–4 h at 37°C until an OD₆₀₀ of 0.4 was reached. The cells were washed twice with 10 mL of LB to remove antibiotics and resuspended in 0.5 mL of LB. Simultaneously, 10 μ l (10⁸) *S. tropica* spores were added to 200 μ l A1 broth and heat shocked at 50°C for 10 min. The spore suspension was mixed with 100 μ l *E. coli* ET12567/pMXT13 and 100 μ l *E. coli* ET12567/pUB307. The mixture was plated on an A1 agar plate and incubated at 30°C for 16–20 hours. Each plate was then overlaid with 1 ml water containing 4 mg nalidixic acid (to selectively kill *E. coli*) and 2 mg kanamycin (for selection of mutants). Following incubation at 30°C for 7–9 days, kanamycin resistant clones were selected, confirmed by PCR and designated as *S. tropica* CNB-4401/*tlm*.

Production, extraction, and detection of salinosporamide A and thiolactomycins.

Five milliliters of A1 medium were inoculated with 10 μ l (10^8) spore suspension of *S. pacifica* CNS-863, *S. tropica* CNB-440, *S. tropica* CNB-4401 or a derivative thereof. The cultures were incubated for 3–4 d at 30 °C at 220 rpm. One milliliter of preculture was inoculated into 50 mL A1 medium using wide bore pipet tips. The cultures were incubated for 9 d at 30 °C with 220 rpm shaking. *S. coelicolor* M1152/*tlm* was cultured as described previously (Tang et al. 2015). The culture supernatant was adjusted to pH 4 with acetic acid and subsequently extracted with an equal volume of ethyl acetate. The organic phase was evaporated, and extracts were dissolved in 0.5 mL methanol (MeOH) and filtered through Acrodisc MS PTFE Syringe filters (Pall Inc., Ann Arbor, MI, USA) prior to HPLC and LC-MS analysis. For HPLC analysis, 10 μ l of the MeOH extract was injected into an Agilent 1200 series analytical HPLC system monitored by UV lamp using a Luna 100A-C18 column (5 μ m, 250 $\times$ 4.6 mm; Phenomenex, US) as follows: 0–23 min, 10–60% B; 23–28 min, 60–100% B; 29–34 min, 100% B; 34–35 min, 100–10% B; 36–40 min, 10% B (solvent A: water/trifluoroacetic acid (999:1); solvent B: acetonitrile/trifluoroacetic acid (999:1)). For LC-MS analysis, 5 μ L of each MeOH extract was injected onto a Phenomenex Luna C18 reverse-phase HPLC column (5 μ m, 250 mm $\times$ 4.6 mm; Phenomenex, US.) and analyzed with an Agilent 6530 Accurate-Mass LC-MS coupled to an Agilent 1260 LC system by gradient elution (A: acetonitrile with 0.1% formic acid (FA); B: H₂O with 0.1% FA: 35–70% A over 23 min, 70–100% A from 23 to 28 min, and 100% A from 28 to 33 min; 0.7 mL/min). Q-TOF MS settings during the LC gradient were as follows: Acquisition—mass range *m/z* 100–1700, MS scan rate 1 s^{−1}, MS/MS scan rate 2 s^{−1}, fixed collision energy 20 eV; Source—gas temperature 300 °C, gas flow 11 L/min; Nebulizer 45 psig, ion polarity positive; Scan source parameters—VCap 3000, Fragmentor 100, Skimmer 65, OctopoleRF Peak 750. The MS was autotuned using Agilent tuning solution in positive mode before each measurement. LC(DAD) data were analyzed with ChemStation software (Agilent), and MS data were analyzed with MassHunter software (Agilent).

Results

Adaptation of the ϕ C31 int/*attP*-*attB* integration system in *S. tropica* CNB-440.

ϕ C31 integrase, a site-specific bacteriophage serine recombinase, catalyzes the insertion of a DNA fragment containing an *attP* site into a chromosomal *attB* site via recombination (Smith et al. 2010). As *attB* sites are widely distributed in a broad range of *Streptomyces* species (Combes et al. 2002; Smith et al. 2004), ϕ C31 int/*attP* has been heavily employed in constructing vectors such as pSET152 (Flett et al. 1997), pCAP01 (Yamanaka et al. 2014), and pCAP03 (Tang et al. 2015) that can be integrated into the chromosome of various *Streptomyces* species for stable maintenance. In contrast to terrestrial *Streptomyces*, marine *Salinispora* species lack the *attB* attachment site in their genomes (Lechner et al. 2011). Previously, we identified three pseudo-*attB* sites in the genome of *S. tropica* CNB-440 that were used to integrate a ϕ C31 phage-based vector carrying a single gene (Lechner et al. 2011). However, despite multiple attempts, we could not obtain exconjugants upon transfer of ϕ C31 phage-based vectors carrying modest or large size BGCs (>30kb) into *S. tropica* CNB-440. This observation is consistent with an earlier study, which reported that insertion

into the *attB* site is 300 times more efficient than into a pseudo-*attB* site (Combes et al. 2002).

We thus decided to introduce an authentic *attB* site into the chromosome of *S. tropica* CNB-440 by RP4-mediated intergeneric conjugation. We first amplified a 500 bp DNA fragment containing the *attB* site from the *gDNA* of *Streptomyces coelicolor* M1152. The PCR product was assembled with a cassette amplified from plasmid pIJ773 (Gust et al. 2003) to obtain an *attB*-containing disruptive cassette with an *attB* site, *aac(3)IV* (Apra^R), *oriT_{RK2}*, and two terminal FLP recognition target (FRT) sequences (Figure S1). This cassette was then cloned into pCRTM2.1-TOPO[®] vector to form pMXT01 (Figure S1). pMXT01 was digested with NotI/SpeI, and the 1,961 bp fragment was purified by agarose gel electrophoresis to eliminate intact plasmid and used as a template for cassette amplification.

Deletion of endogenous BGCs has been used as a general strategy to 1) increase the metabolic flux of precursor availability and productivity of heterologously expressed BGCs, and 2) simplify the identification and characterization of heterologous products by activity screening and metabolite profiling (Gomez-Escribano and Bibb 2011; Komatsu et al. 2010). Thus, the fosmid BHXS1782 (Eustaquio et al. 2009) with a 44 kb insert containing the salinosporamide BGC (*sal*) was chosen to carry the *attB*-containing disruptive cassette into *S. tropica* CNB-440 to generate a double crossover mutant. We first replaced assembly line biosynthesis genes *salA* to *salC* on the fosmid BHXS1782 with the *attB*-containing disruptive cassette by Red/ET-mediated recombination, generating the fosmid pMXT04 (Figure 1). Subsequently, the region of *aac(3)IV* (Apra^R) plus *oriT_{RK2}* was eliminated *in vivo* using FLP-mediated recombination to form pMXT10 (Figure 1). Additionally, the cassette containing *aac(3)IV* (Apra^R) plus *oriT_{RK2}* sequence was used to replace the chloramphenicol resistance gene *cat*, as *aac(3)IV* is a better selection marker for generating single crossover mutants in *Salinispora* and *oriT_{RK2}* is essential for *E. coli*-mediated conjugation. The resulting fosmid, pMXT11, was introduced into *S. tropica* CNB-440 by conjugation (Figure S2). Single crossover mutants were selected for their apramycin resistance. Intergeneric conjugation between *E. coli* ET12567/pUZ8002/pMXT11 and *S. tropica* CNB-440 produced 10–20 colonies with apramycin resistance per conjugation using 10 µl (10⁸) *S. tropica* spores. Although the efficiency is much lower than for conjugation with *Streptomyces* spores (10⁴–10⁵ using 10⁸ spores), this might be due to the high concentration of sea salt in the A1 agar plate, which is essential for the growth of *S. tropica*.

In order to obtain a double crossover mutant, single crossover mutants were streaked for single colonies on A1 agar plates without antibiotics. Fifty colonies were tested for apramycin sensitivity, and strains no longer resistant to apramycin were obtained for further verification. Total DNA was isolated from selected colonies, and a pair of primers flanking the outside of *salA-C* was designed for PCR confirmation. As designed, amplification of a double crossover deletion mutant gave a 626 bp PCR product (Figure 2). Furthermore, sequencing of the PCR product confirmed the presence of the *attB* site in the mutant strain, which was designated *S. tropica* CNB-4401. In the case of wild-type *S. tropica* CNB-440, no PCR products were obtained, as the targeted region was too large (9830 bp) (Figure 2).

We also compared the production of salinosporamide A, the major product of the *sal* BGC from *S. tropica* CNB-440 and *S. tropica* CNB-4401. Because *salA-C* code for the salinosporamide enzymatic assembly line, *S. tropica* CNB-4401 should lose all ability to produce salinosporamide A. After cultivation, extracts were subjected to high resolution LC-ESI-MS analysis. In contrast to the wild-type strain *S. tropica* CNB-440, production of salinosporamide A (Figure 2) and all other analogs was completely abolished in the double crossover deletion mutant *S. tropica* CNB-4401. This result further confirmed the successful replacement of *salA-C* by the *attB*-containing disruptive cassette in the strain *S. tropica* CNB-4401. Additionally, the HPLC chromatogram of the ethyl acetate extract of the culture supernatant from *S. tropica* CNB-4401 contains a small number of peaks and a low baseline under various UV detection wavelengths (Figure S3). This would simplify downstream processes in detection and purification of the heterologously produced metabolites.

Heterologous expression of an exogenous biosynthetic gene cluster in *S. tropica* CNB-4401.

In order to assess the performance of *S. tropica* CNB-4401 as a heterologous host in expressing large BGCs, we selected the recently characterized BGC for the fatty acid synthase inhibitor thiolactomycin (*tlm*) that is only present in strains of *S. pacifica*. Other high-profile BGCs from *Salinispora*, such as the salinosporamide, lymphostin, and lomaiviticin BGCs, are endogenous to *S. tropica* CNB-440 and therefore were not selected for host validation. The *tlm* BGC encodes an unconventional polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) hybrid assembly line, which represents an important class of natural products (Tang et al. 2015). Furthermore, the *tlm* genes were previously cloned into the integration vector pCAP03 to generate pMXT13 and successfully expressed in the *Streptomyces coelicolor* M1152 “superhost,” albeit at lower levels than the wild type producer (Gomez-Escribano and Bibb 2011). Thus, the *tlm* BGC was directly introduced to the *S. tropica* CNB-4401 host via integration into the newly introduced genomic ϕ C31 *attB* site. Kanamycin resistant clones were selected and designated *S. tropica* CNB-4401/*tlm*. Intergeneric conjugation between *E. coli* ET12567/pUB307 (helper), *E. coli* ET12567/pMXT13, and *S. tropica* CNB-4401 yielded around 5 to 10 Kan^R exconjugants (experiment in sextuplicate) per conjugation using 10 μ L (10^8) *S. tropica* CNB-4401 spores (Figure S4).

Although the integration efficiency is again much lower than for *Streptomyces* spores, our data are comparable with the allelic exchange rate for making single crossover mutants of *S. tropica* CNB-440 (10–20 mutants by biparental conjugation). This data thus supports the hypothesis that conjugation efficiency may be hampered by high salt concentrations necessary for the growth of some marine bacteria. No exconjugants were obtained from identical attempts to integrate the *tlm* BGC into the genome of *S. tropica* CNB-440 (Figure S4, experiment in sextuplicate). Therefore, our results strongly indicate that *S. tropica* CNB-4401 is capable of being readily integrated with large exogenous DNA fragments using the ϕ C31 int/attP-attB integration system, while *S. tropica* CNB-440 is not. This strategy may be widely applicable in engineering other bacteria as integratable heterologous hosts that are directly compatible with established ϕ C31-containing vectors (Martinez et al. 2004).

With the successful integration of the *tlm* biosynthetic pathway in *S. tropica* CNB-4401, we cultured, extracted, and analyzed the supernatant extracts of the *S. tropica* CNB-4401/*tlm* heterologous host by HPLC and LC-MS. The production of thiolactomycin (**1**) and three analogues (10-methyl thiolactomycin (**2**), 11-methyl thiolactomycin (**3**), and thiotetromycin (**4**)) were identified from the extracts of *S. tropica* CNB-4401/*tlm* by comparison with thiolactomycins produced by the native producer *S. pacifica* CNS-863 as well as the heterologous producer *S. coelicolor* M1152/*tlm*. All four thiolactomycin analogues were previously fully characterized by 1D and 2D NMR experiments (Figure 3). Corresponding parent mass peaks for (**1**) with m/z 211.0789 $[M+H]^+$ (calcd for $C_{11}H_{15}O_2S$, 211.0793), (**2**) with m/z 225.0944 $[M+H]^+$ (calcd for $C_{12}H_{17}O_2S$, 225.0949), (**3**) with m/z 225.0942 $[M+H]^+$ (calcd for $C_{13}H_{19}O_2S$, 225.0949), and (**4**) with m/z 239.1097 $[M+H]^+$ (calcd for $C_{12}H_{17}O_2S$, 239.1106) were detected by high resolution LC-MS (Figure S5). Thus, this is the first report of using the marine actinomycete *Salinispora* as an integratable host for natural product BGC heterologous expression.

In contrast to *S. coelicolor* M1152/*tlm*, the production of thiolactomycin (**1**) from *S. tropica* CNB-4401/*tlm* and *S. pacifica* CNS-863 was approximately 3-fold higher (Figure 3), while production of **3** and **4** were only slightly lower. Overall, the production pattern of the four thiolactomycin analogues in *S. tropica* CNB-4401/*tlm* was very similar to that of the native producer, *S. pacifica* CNS-863, and subtly different from *S. coelicolor* M1152 (Figure 3). This difference in production levels as well as PKS extender unit incorporation suggests that heterologous host context with respect to genetics, biochemistry, and metabolism, influences heterologous thiolactomycin production and may also influence heterologous production of products associated with other BGCs.

Discussion

In this study, we selected *S. tropica* CNB-440 as the starting point for developing the first marine actinomycete heterologous host. In order to establish an integratable expression host with a cleaner background for convenient metabolic profiling, we introduced a disruptive cassette containing the well-established Φ C31 phage attachment site *attB* to replace three essential salinisporamide biosynthetic genes *sala*–*C* in *S. tropica* CNB-440. This newly established mutant strain *S. tropica* CNB-4401, therefore, possesses an authentic *attB* attachment site to integrate large exogenous BGCs through Φ C31 integrase activity for heterologous expression. We validated this new heterologous host with the successful integration and expression of the thiolactomycin BGC from several *S. pacifica* strains. To the best of our knowledge, this is the first example of using a marine actinomycete as a heterologous host for BGC expression.

The overall yield of all thiolactomycins produced heterologously by *S. tropica* CNB-4401 is very similar to the yield from the native producer *S. pacifica* CNS-863 and approximately 3 times higher than the yield from *S. coelicolor* M1152 (Figure 3). This is despite the fact that the *S. coelicolor* M1152 “superhost” has been subjected to about a dozen rounds of genetic manipulation to delete endogenous pathways and introduce a point mutation to *rpoB* that has been empirically shown to enhance antibiotic production in *Streptomyces* (Gomez-Escribano and Bibb 2011).

The different ratios of compounds **1–4** observed between *S. tropica* CNB-4401/*tlm* (or *S. pacifica* CNS-863) and *S. coelicolor* M1152/*tlm* may be due to differences in methylmalonyl-CoA and ethylmalonyl-CoA precursor supply in these strains. However, previous gene deletion and heterologous expression experiments in *S. coelicolor* M1152 suggest that the pool of ethylmalonyl-CoA precursors used by the TlmH PKS to generate compounds **2–4** is primarily supplied by the pathway specific crotonyl-CoA carboxylase (TlmB) and 3-hydroxybutyryl-CoA dehydrogenase (TlmC) (Tang et al. 2017). Thus, there may be additional mechanisms beyond precursor supply that influence TlmH precursor utilization, which results in a different profile of thiolactomycin production levels in *S. coelicolor* M1152 compared to *S. tropica* CNB-4401 and *S. pacifica* CNS-863. This supports our previous finding that host context can play an important role in heterologous compound production and provide biosynthetic insights (Zhang et al. 2017a).

S. tropica expresses the unusual crotonyl-CoA carboxylase SalG that not only catalyzes the construction of ethylmalonyl-CoA, but also the unique PKS precursor chloroethylmalonyl-CoA (Eustaquio et al. 2009) and the rare propylmalonyl-CoA (Liu et al. 2009). We employed LC-MS to interrogate thiolactomycin analogues that may have incorporated these alternate substrates, but no new thiolactomycin analogues were identified, perhaps due to substrate selectivity of the *tlm* PKS assembly line. Alternatively, insertion of a large DNA fragment into the engineered attachment site, which is upstream of the crotonyl-CoA carboxylase SalG, may cause polar effects that disrupt expression of *salG*. Heterologous expression of large BGCs remains challenging, particularly for pathways that require rare precursors and/or utilize uncertain regulatory, biosynthetic, or resistance factors. Our previous work to develop improved direct cloning and heterologous expression vectors demonstrated that having access to genetically diverse heterologous host organisms will enhance the likelihood of achieving successful heterologous expression for biosynthetic studies and identification of new compounds (Zhang et al. 2017a). As *S. tropica* CNB-4401 is a phylogenetically distinct actinomycete host that can now be easily leveraged using existing *Streptomyces* expression vectors for heterologous expression experiments, we believe that our study provides the natural product community an alternative and facile platform for discovering and engineering BGCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic diagram depicting (A) the replacement of *salA-C* by Red/ET-mediated recombination with the *attB*-containing cassette; (B) subsequent removal of the resistance marker by FLP-mediated excision; and (C) replacement of the fosmid backbone chloramphenicol resistant gene *cat* with *acc(3)IV* (Apra^R) and *oriT* of RK2 to generate fosmid pMXT11 to simultaneously knock-in the *attB* attachment site and knock-out *salA-C* in *S. tropica* CNB-440.

Figure 2.
Verification of *S. tropica* CNB-4401 by (A) PCR amplification and (B) loss of production of salinosporamide A observed by LC-MS.

Figure 3.

Detection of thiolactomycin and its analogues in various producing strains by HPLC. (A) Schematic illustrating the thiolactomycin (*tlm*) biosynthetic gene cluster. HPLC chromatograms of ethyl acetate extracts from (B) *S. tropica* CNB-4401/*tlm*, (C) *S. tropica* CNB-4401, (D) *S. pacifica* CNS-863, and (E) *S. coelicolor* M1152/*tlm*. Detection at 239 nm. (F) Structures of thiolactomycin (1), 10-methyl thiolactomycin (2), 11-methyl thiolactomycin (3), and thiotetromycin (4).

Table 1.

Plasmids and bacterial strains used in this work

Plasmid/Strains	Description	References
Plasmids		
BHXS1782	pCC1FOS fosmid that carries a 44 kb genomic region containing the entire salinosporamide BGC from <i>S. tropica</i> CNB-440	Eustaquio et al. 2009
pMXT13	pCAP03 derivative that carries a 26 kb genomic region containing the entire thiolactomycin BGC from <i>S. pacifica</i> CNS-863	Tang et al. 2015
pIJ773	A plasmid containing the cassette <i>aac(3)IV</i> (Apra ^R) + <i>oriT</i>	Gust et al. 2003
pKD20	λ -RED (<i>gam</i> , <i>bet</i> , <i>exo</i>), <i>bla</i> , <i>araC</i> , <i>oriR101</i> , <i>rep101ts</i>	Datsenko and Wanner 2000
pUZ8002	<i>neo</i> , RP4	Paget et al. 1999
pUB307	Self-transmissible plasmid that mobilizes other plasmids in trans for DNA transfer into hosts: RP4, <i>neo</i>	Flett et al. 1997
pMXT01	<i>acc(3)IV</i> , <i>PermE</i> , FRT, <i>bla</i>	This study
pMXT04	BHXS1782 derivative in which <i>salA-C</i> has been replaced by the knockout cassette from pMXT01	This study
pMXT10	pMXT04 derivative in which <i>acc(3)IV</i> (Apra ^R) has been removed by FLP-FRT recombination	This study
pMXT11	pMXT10 derivative in which chloramphenicol resistance gene <i>cat</i> has been replaced by the cassette containing <i>oriT</i> and <i>aac(3)IV</i> (Apra ^R) from pIJ773	This study
Strains		
<i>S. tropica</i> CNB-440	Wild-type, ATCC BAA-916 / DSM 44818	Udwary et al. 2007
<i>S. tropica</i> CNB-4401	<i>S. tropica</i> CNB-440 derivative, <i>sal</i> and Φ C31 <i>attB</i>	This study
<i>S. tropica</i> CNB-4401/ <i>tlm</i>	<i>S. tropica</i> CNB-4401 derivative, <i>sal</i> and Φ C31 <i>attB</i> with <i>tlm</i> integration	This study
<i>E. coli</i> Top10	Host strain for routine cloning: F-, (<i>araA-leu</i>)7697, (<i>araD139</i>)B/r, (<i>codB</i> lacI)3, ϕ 80d <i>lacZ</i> 58(M15), <i>galK0</i> , <i>mcrA0</i> , <i>galU</i> , <i>recA1</i> , <i>endA1</i> , <i>nupG</i> , <i>rpsL</i> -(<i>strR</i>), (<i>mcrC-mrr</i>)715	Invitrogen
<i>E. coli</i> BW25113	Host strain for λ -RED recombination, K12 derivative: F-, (<i>araD</i> araB)567, <i>lacZ</i> 4787::rrnB-3), λ -, <i>rph</i> -1, (<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	Datsenko and Wanner 2000
<i>E. coli</i> BT340	Host strain for FLP recombination: F-, (<i>argF-lac</i>)169, ϕ 80d <i>lacZ</i> 58(M15), <i>glnV44</i> (AS), λ -, <i>rfbC1</i> , <i>gyrA96</i> (NalR), <i>recA1</i> , <i>endA1</i> , <i>spoT1</i> , <i>thi</i> -1, <i>hsdR</i> 17, pCP20	Cherepanov and Wackernagel 1995
<i>E. coli</i> ET12567	DNA methylation deficient donor strain for conjugation: F2 <i>dam</i> 13::Tn9, <i>dcm</i> , <i>6 hsdM</i> , <i>hsdR</i> , <i>recF</i> 143, <i>zji</i> -202::Tn10, <i>galK2</i> , <i>galT</i> 22 <i>ara</i> -14, <i>laacY1</i> , <i>xyl</i> -5, <i>leuB6</i> , <i>thi</i> -1, <i>tonA31</i> , <i>rpsL</i> 136, <i>hisG4</i> , <i>tsx</i> -78, <i>mtl</i> -1, <i>glnV44</i>	Flett et al. 1997