JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

Direct Capture and Heterologous Expression of *Salinispora* Natural Product Genes for the Biosynthesis of Enterocin

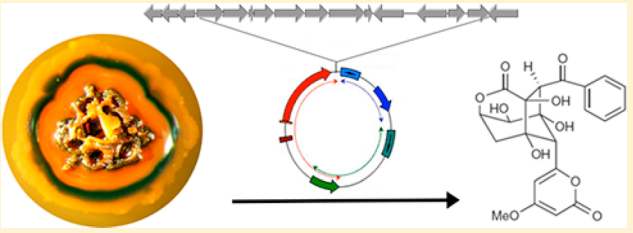
Bailey Bonet,[†] Robin Teufel,[†] Max Crüsemann,[†] Nadine Ziemert,*[,†] and Bradley S. Moore*[,†,‡]

[†]Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, La Jolla, California 92037, United States

[‡]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California 92093, United States

Supporting Information

ABSTRACT: Heterologous expression of secondary metabolic pathways is a promising approach for the discovery and characterization of bioactive natural products. Herein we report the first heterologous expression of a natural product from the model marine actinomycete genus *Salinispora*. Using the recently developed method of yeast-mediated transformation-associated recombination for natural product gene clusters, we captured a type II polyketide synthase pathway from *Salinispora pacifica* with high homology to the enterocin pathway from *Streptomyces maritimus* and successfully produced enterocin in two different *Streptomyces* host strains. This result paves the way for the systematic interrogation of *Salinispora*'s promising secondary metabolome.



With the increased ease in obtaining next-generation DNA sequencing information, bioinformatic analyses and genome mining methods have become important modern tools in the discovery of new microbial natural products.[1] A recent survey of all publicly available genomes revealed over 30 000 biosynthetic gene clusters that potentially allow the production of novel bioactive small molecules.[2] This genetic reservoir represents a readily available resource for biosynthetic discovery and inspiration. However, the identification and characterization of the associated compounds can be challenging due to immense variations in microbial culturing and secondary metabolite production under normal laboratory conditions. A number of analytical methods have been developed in recent years to overcome these vexing problems and efficiently connect genes and gene clusters to small molecules.[3−6] One of the most promising approaches involves the heterologous expression of entire biosynthetic gene clusters in a reliable and genetically tractable host organism. Heterologous expression not only allows the production of small molecules independent of growth rate and culturability of the natural producer, but also enables genetic manipulations to enhance compound production levels, the discovery of new biosynthetic enzymes, or engineering of unnatural biosynthetic products.[7]

So far, no compatible heterologous host system has been reported for the obligate marine actinomycete *Salinispora*.[8] This genus is known to produce a wealth of bioactive natural products of often unparalleled chemical structures[9,10] and has been found to remarkably devote approximately 10% of its genome to their production.[11] Recent bioinformatic efforts allowed the detection of more than 100 orphan pathways associated with polyketide and nonribosomal peptide biosynthesis in 75 newly sequenced *Salinispora* strains.[12] Previous genome mining studies in *Salinispora* connected orphan gene clusters to salinilactam A,[11] salinosporamide K,[13] and the lomaiviticins,[14] yet in each of these cases, production was limited to the native strain. Thus, the goal of this study was to develop an efficient heterologous expression system to provide a key tool for interrogating the biosynthesis of new natural products by these chemically prolific marine actinobacteria.

Common genetic tools and methods used with terrestrial actinomycetes work well for *in vivo* manipulations in *Salinispora* species.[15,16] The 70% average GC content of *Salinispora* genomes is very similar to the well-established *Streptomyces* superhosts widely used for the expression of actinomycete pathways.[17] Therefore, we decided to test the suitability of two common *Streptomyces* host strains, the genome-minimized *Streptomyces coelicolor* M1146 and *Streptomyces lividans* TK23,[18,19] for the production of *Salinispora* compounds. Of the more than 100 orphan biosynthetic pathways identified in an earlier study,[12] we selected an 18 kb type II PKS gene cluster from *Salinispora pacifica* CNT-150 that showed high homology to the enterocin locus in *Streptomyces maritimus* for our proof of principle study (Table 1).[20]

Enterocin (**1**) is a bacteriostatic polyketide with a highly unusual structure containing a caged tricyclic, nonaromatic core whose formation involves a Favorskii-like oxidative rearrangement of a reactive poly(β-carbonyl) substrate catalyzed by the unique flavoenzyme EncM.[21] Previously, enterocin and its

Special Issue: Special Issue in Honor of William Fenical

Received: August 21, 2014
Published: November 10, 2014

Table 1. Enterocin Gene Cluster in *Salinispora pacifica* CNT-150

	JGI gene locus	gene product	homologue	identity	accession no.
1	B174DRAFT_04104	hypothetical protein	EncG, *S. maritimus*	59%	AAF81722.1
2	B174DRAFT_04105	methyltransferase	EncK, *S. maritimus*	61%	AAF81726.1
3	B174DRAFT_04106	ketoreductase	EncD, *S. maritimus*	66%	AAF81727.1
4	B174DRAFT_04107	3-oxoacyl-(acyl-carrier-protein) synthase, α subunit	EncA, *S. maritimus*	72%	AAF81728.1
5	B174DRAFT_04108	3-oxoacyl-(acyl-carrier-protein) synthase, β subunit	EncB, *S. maritimus*	62%	AAF81729.1
6	B174DRAFT_04109	acyl carrier protein	EncC, *S. maritimus*	37%	AAF81730.1
7	B174DRAFT_04110	acyl transferase	EncL, *S. maritimus*	51%	AAF81731.1
8	B174DRAFT_04111	FAD-dependent favorskiiase	EncM, *S. maritimus*	64%	AAF81732.1
9	B174DRAFT_04112	cytochrome P450, monooxygenase	EncR, *S. maritimus*	49%	AAF81737.1
10	B174DRAFT_04113	phenylalanine ammonia-lyase	EncP, *S. maritimus*	64%	AAF81735.1
11	B174DRAFT_04114	hypothetical protein	EncO, *S. maritimus*	44%	AAF81734.1
12	B174DRAFT_04115	benzoyl-CoA ligase	EncN, *S. maritimus*	46%	AAF81733.1
13	B174DRAFT_04117	drug resistance transporter, EmrB/QacA subfamily	EncT, *S. maritimus*	57%	AAF81738.1
14	B174DRAFT_04118	transcriptional regulator	EncS, *S. maritimus*	58%	AAF81739.1
15	B174DRAFT_04119	transcriptional activator	hypothetical protein [*S.* sp. MspMP-M5]	37%	WP_018542724.1
16	B174DRAFT_04120	acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II	long-chain-fatty-acid-CoA ligase [*B. saxobsidens* DD2]	49%	YP_005329444.1

structural analogues were heterologously expressed in *S. lividans*, thereby confirming the function and entirety of the *enc* locus in *S. maritimus*.[22]

Sequence comparisons of both *enc* gene clusters revealed that all essential enzymes involved in enterocin biosynthesis in *S. maritimus* are also present in the *S. pacifica* pathway,[23] although with somewhat different gene arrangement (Figure 1, Table 1).

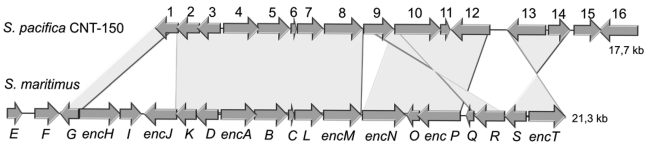


Figure 1. Comparison of the enterocin (*enc*) gene clusters of *Streptomyces maritimus* and *Salinispora pacifica* CNT-150. Numbered genes in *S. pacifica* are further described in Table 1.

Genes missing from the *S. pacifica enc* locus are *encH*–*J*, which encode beta-oxidation enzymes involved in starter unit processing, and *encQ*, which codes for a ferredoxin cofactor, all of which were previously shown to be complemented by primary metabolic enzymes.[24] Two additional genes flanking the *S. pacifica enc* cluster, *enc*15 and *enc*16, showed no homology to those in *S. maritimus* and are predicted to code for a transcription factor and an acyl-CoA synthetase, respectively.

To test whether the enterocin gene cluster in *S. pacifica* CNT-150 is active under laboratory conditions, extracts from 7-day-old cultures were tested by HR-LCMS and compared to an enterocin standard (Figure 2). Mass and UV data of one of the prominent peaks in the tested extracts indeed fully matched enterocin, which was furthermore verified by NMR analysis of the purified compound (Figure S1). *S. pacifica* CNT-150 is thus the first bacterium outside the *Streptomyces* genus shown to produce enterocin.

To express a selected microbial metabolite such as enterocin in a *Streptomyces* host, it is necessary to clone the gene cluster in an appropriate integrative vector, a process that usually involves time-consuming genomic library and cloning efforts. To apply a more direct cloning method, we employed the pCAP01 vector that we recently designed for the transformation-associated

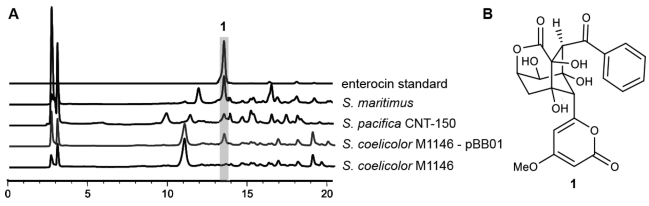


Figure 2. Heterologous expression of enterocin. (A) HPLC chromatograms of extracts of the heterologous host *Streptomyces coelicolor* M1146 with and without the expression plasmid pBB01 as well as the natural producer strains *Streptomyces maritimus* and *Salinispora pacifica* CNT-150. Detection at 254 nm. (B) Structure of enterocin.

recombination (TAR) of biosynthetic pathways in yeast directly from genomic DNA.[25,26] The method was used to capture and express marinopyrrole from *Streptomyces* sp. CNQ-418 and taromycin A from *Saccharomonospora* sp. CNQ-490 in a *S. coelicolor* host strain. Hence we reasoned that this method seemed well suited for *Salinispora* as well as other actinomycete genera.

We captured by TAR a 21.3 kb region of genomic DNA containing the enterocin gene cluster from *S. pacifica* CNT-150 and subsequently transferred the vector through yeast and *Escherichia coli* to the *Streptomyces* host strains. Extracts of positive clones were tested for enterocin production after 6 days of growth by HR-LCMS (Figure 2). Indeed, enterocin production could be detected in all tested *Streptomyces* clones containing the pathway. No significant differences in production levels for both *Streptomyces* host species were detected, proving that the genus is principally well suited for the heterologous expression of *Salinispora* biosynthetic pathways (Figure S5).

The enterocin gene cluster in *S. maritimus* has been shown to be a highly versatile biosynthetic system that produces a large series of metabolites, including 5-deoxyenterocin, 3-epi-5-deoxyenterocin, and different wailupemycin analogues.[20] The latter are assumed to arise from spontaneous cyclization of the extremely unstable EncM substrate, which competes with the enzymatic Favorskii rearrangement. Further enterocin ana-

dx.doi.org/10.1021/np500664q | *J. Nat. Prod.* 2015, 78, 539–542

logues have been isolated from marine ascidians.[27] The lipoesters enterocin-arachidate and -behenate were speculated to be produced by uncultivated bacterial symbionts of a Western Australian *Didemnum* species. To test the spectrum of enterocin analogues produced by the *S. pacifica* cluster, we employed mass spectral molecular networking[28] to compare the metabolome of both natural producers and one heterologous host (Figure S6). Although mass spectra of known analogues such as 5′-deoxyenterocin could be observed, no new enterocin or wailupemycin derivatives were detected in the network. Notably, wailupemycins were completely absent in extracts of both *S. pacifica* CNT-150 and the heterologous host strain. Why these spontaneous cyclization products are not produced by *S. pacifica* CNT-150 can only be speculated and might suggest a more controlled substrate channeling by the early enterocin biosynthetic machinery (EncABCDM). Gene deletion experiments of the superfluous acyl-CoA synthetase gene *enc*16 in the heterologous producer *S. coelicolor* M1146 showed no effects on the production of enterocin and related compounds, thereby implying that the enzyme is not involved in the biosynthesis of alternative derivatives.

Of the 75 sequenced *Salinispora* genomes presently available in public databases, only two additional strains contain the enterocin pathway, the Hawaiian isolates *S. pacifica* CNT-851 and *S. pacifica* CNT-796. Although these strains belong to a different phylotype[29] and originate from a different location than *S. pacifica* CNT-150, which was isolated in Fiji, the nucleotide sequence is nearly identical in all three strains. This relationship suggests a very recent event of horizontal gene transfer, as has been demonstrated for the majority of secondary metabolite pathways in this highly diverse species.[12]

In conclusion, *in silico* analyses and comparative genomics have shown that the marine actinomycete genus *Salinispora* contains among the highest diversity of secondary metabolite classes within the biosynthetic rich group of actinomycetes.[30] The recent genome sequencing of 75 *Salinispora* genomes furthermore revealed a high number and variety of orphan pathways in closely related strains,[12] suggesting an enormous untapped potential of novel small molecules. With this study we show for the first time the heterologous expression of a *Salinispora* natural product pathway. Because heterologous expression and genetic manipulations are well established in *Streptomyces*, this study sets the stage for a more systematic approach to access the available genetic potential of *Salinispora*. Using TAR cloning to directly capture the gene clusters from genomic DNA offers a fast and efficient way for production and genetic manipulations, thus allowing rapid and detailed investigation of promising biosynthetic pathways.

EXPERIMENTAL SECTION

Bacterial Strains, Plasmids, and Culture Conditions. All strains used are listed in Table S1. *E. coli* cultures were grown in LB liquid media or on LB agar plates with antibiotics when necessary (50 μg/mL kanamycin, 50 μg/mL apramycin, 25 to 50 μg/mL chloramphenicol, 50 μg/mL carbenicillin) at 37 °C with shaking. *Streptomyces* liquid precultures were grown in tryptic soy broth with the appropriate antibiotics (50 μg/mL kanamycin, 50 μg/mL apramycin, 25 μg/mL nalidixic acid), inoculated into SSM media (1% soytone, 1% soluble starch, 2% maltose, 0.5% trace element solution, pH 5.7) and grown at 30 °C with shaking. *Streptomyces* were also grown on mannitol soya flour medium plates (2.0% agar, 2.0% mannitol, 2.0% soya flour) with appropriate antibiotics (50 μg/mL kanamycin, 50 μg/mL apramycin, 25 μg/mL nalidixic acid). *Salinispora* were grown in A1 media (1.0% starch, 0.4% yeast extract, 0.2% peptone, and 2.8% sea salt) at 30 °C with shaking.

TAR Capture of the *Salinispora pacifica enc* Gene Cluster. The method of TAR capturing used in this study has been previously reported.[25] The isolation of high-quality genomic DNA from *Salinispora* was performed according to standard procedures.[31] The capture vector, pCAP01, containing yeast ARSH4/CEN6 and TRP1 marker, *E. coli pUC ori*, *Streptomyces* φC31 integrase gene(int), its attachment site (attP) and origin of DNA transfer (*oriT*), aph(3)II gene (Kan/Neo resistance), and two 1 kb regions flanking the gene cluster on either side was used to capture and propagate the enterocin gene cluster. The vector pCAP01 with the captured *enc* gene cluster from *S. pacifica* CNT-150 was named pBB01.

Heterologous Expression, Extraction, and HPLC Analysis. The plasmid pBB01 and its derivatives were conjugated into *S. lividans* TK23 and *S. coelicolor* M1146 using a standard triparental mating method with *E. coli* ET 12567/pBB01 and *E. coli* ET 12567/pUB307.[31] After 6 days of growth in SSM media, the cultures were extracted with four volumes of EtOAc and concentrated *in vacuo*. The extracts were then dissolved in 100 μL of MeCN and analyzed by HPLC using a Luna 5u C_{18} column (150 mm × 4.60 mm, 5 μm beads) with a linear gradient of 2−100% MeCN in water with 0.1% TFA over 30 min with a flow rate of 0.7 mL/min.

HPLC-MS/MS and Molecular Networking. HPLC-HR-ESI-MSMS analysis of all extracts was carried out on an Agilent 1290 Q-TOF (200−2000 *m/z*, 20 keV). The data were subjected to the molecular networking workflow and analyzed as described previously.[36]

Gene Deletion Experiment. The inactivation of the acyl-CoA synthetase encoding gene, *enc16*, in the heterologous host strain *S. coelicolor* M1146-pBB01 was done using λ-Red recombination with an apramycin-resistant marker (aac(3)IV) as described previously.[32]

ASSOCIATED CONTENT

Supporting Information

NMR spectra for **1**, molecular network of *Salinispora pacifica*, *Streptomyces maritimus*, and *Streptomyces coelicolor* extracts, and detailed information about the strains and plasmids used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: nziemert@ucsd.edu.
*E-mail: bsmoore@ucsd.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank P. R. Jensen and M. J. Bibb for providing bacterial strains *S. pacifica* CNT-150 and *S. coelicolor* M1146, respectively. We also thank B. M. Duggan for assistance with NMR, and X. Tang and P. C. Dorrestein for helpful discussions about λ-Red recombination techniques and molecular networking. This work was supported by NIH grants AI-47818 and GM-85770 to B.S.M. and DFG postdoctoral fellowships to R.T. and M.C.

DEDICATION

Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of California−San Diego, for his pioneering work on bioactive natural products.

REFERENCES

(1) Jensen, P. R.; Chavarria, K. L.; Fenical, W.; Moore, B. S.; Ziemert, N. *J. Ind. Microbiol. Biotechnol.* **2014**, *41*, 203−209.
(2) Cimermancic, P.; Medema, M. H.; Claesen, J.; Kurita, K.; Wieland Brown, L. C.; Mavrommatis, K.; Pati, A.; Godfrey, P. A.; Koehrsen, M.; Clardy, J.; Birren, B. W.; Takano, E.; Sali, A.; Linington, R. G.; Fischbach, M. A. *Cell* **2014**, *158*, 412−421.
(3) Kersten, R. D.; Yang, Y.-L.; Xu, Y.; Cimermancic, P.; Nam, S.-J.; Fenical, W.; Fischbach, M. A.; Moore, B. S.; Dorrestein, P. C. *Nat. Chem. Biol.* **2011**, *7*, 794−802.
(4) Kersten, R. D.; Ziemert, N.; Gonzalez, D. J.; Duggan, B. M.; Nizet, V.; Dorrestein, P. C.; Moore, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, E4407−4416.
(5) Gross, H.; Stockwell, V. O.; Henkels, M. D.; Nowak-Thompson, B.; Loper, J. E.; Gerwick, W. H. *Chem. Biol.* **2007**, *14*, 53−63.
(6) Winter, J. M.; Behnken, S.; Hertweck, C. *Curr. Opin. Chem. Biol.* **2011**, *15*, 22−31.
(7) Ongley, S. E.; Bian, X.; Neilan, B. A.; Müller, R. *Nat. Prod. Rep.* **2013**, *30*, 1121−1138.
(8) Maldonado, L. A.; Fenical, W.; Jensen, P. R.; Kauffman, C. A.; Mincer, T. J.; Ward, A. C.; Bull, A. T.; Goodfellow, M. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1759−1766.
(9) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Angew. Chem., Int. Ed.* **2003**, *42*, 355−357.
(10) Jensen, P. R.; Williams, P. G.; Oh, D. C.; Zeigler, L.; Fenical, W. *Appl. Environ. Microbiol.* **2007**, *73*, 1146−1152.
(11) Udwary, D. W.; Zeigler, L.; Asolkar, R. N.; Singan, V.; Lapidus, A.; Fenical, W.; Jensen, P. R.; Moore, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10376−10381.
(12) Ziemert, N.; Lechner, A.; Wietz, M.; Millán-Aguiñaga, N.; Chavarria, K. L.; Jensen, P. R. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, E1130−1139.
(13) Eustáquio, A. S.; Nam, S. J.; Penn, K.; Lechner, A.; Wilson, M. C.; Fenical, W.; Jensen, P. R.; Moore, B. S. *ChemBioChem* **2011**, *12*, 61−64.
(14) Kersten, R. D.; Lane, A. L.; Nett, M.; Richter, T. K. S.; Duggan, B. M.; Dorrestein, P. C.; Moore, B. S. *ChemBioChem* **2013**, *14*, 955−962.
(15) Eustáquio, A. S.; Pojer, F.; Noel, J. P.; Moore, B. S. *Nat. Chem. Biol.* **2008**, *4*, 69−74.
(16) Lechner, A.; Eustáquio, A. S.; Gulder, T. A. M.; Hafner, M.; Moore, B. S. *Chem. Biol.* **2011**, *18*, 1527−1536.
(17) Baltz, R. H. *J. Ind. Microbiol. Biotechnol.* **2010**, *37*, 759−772.
(18) Gomez-Escribano, J. P.; Bibb, M. J. *Microb. Biotechnol.* **2011**, *4*, 207−215.
(19) Penn, J.; Li, X.; Whiting, A.; Latif, M.; Gibson, T.; Silva, C. J.; Brian, P.; Davies, J.; Miao, V.; Wrigley, S. K.; Baltz, R. H. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 121−128.
(20) Piel, J.; Hertweck, C.; Shipley, P. R.; Hunt, D. M.; Newman, M. S.; Moore, B. S. *Chem. Biol.* **2000**, *7*, 943−955.
(21) Teufel, R.; Miyanaga, A.; Michaudel, Q.; Stull, F.; Louie, G.; Noel, J. P.; Baran, P. S.; Palfey, B.; Moore, B. S. *Nature* **2013**, *503*, 552−556.
(22) Piel, J.; Hoang, K.; Moore, B. S. *J. Am. Chem. Soc.* **2000**, *122*, 5415−5416.
(23) Cheng, Q.; Xiang, L.; Izumikawa, M.; Meluzzi, D.; Moore, B. S. *Nat. Chem. Biol.* **2007**, *3*, 557−558.
(24) Xiang, L.; Moore, B. S. *J. Bacteriol.* **2003**, *185*, 399−404.
(25) Yamanaka, K.; Reynolds, K. A.; Kersten, R. D.; Ryan, K. S.; Gonzalez, D. J.; Nizet, V.; Dorrestein, P. C.; Moore, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 1957−1962.
(26) Ross, A. C.; Gulland, L. E. S.; Dorrestein, P. C.; Moore, B. S. *ACS Synth. Biol.* **2014**, DOI: 10.1021/sb500280q.
(27) Kang, H.; Jensen, P. R.; Fenical, W. *J. Org. Chem.***1996**, *61*, 1543−1546.
(28) Watrous, J.; Roach, P.; Alexandrov, T.; Heath, B. S.; Yang, J. Y.; Kersten, R. D.; van der Voort, M.; Pogliano, K.; Gross, H.; Raaijmakers, J. M.; Moore, B. S.; Laskin, J.; Bandeira, N.; Dorrestein, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, E1743−E1752.
(29) Freel, K. C.; Millán-Aguiñaga, N.; Jensen, P. R. *Appl. Environ. Microbiol.* **2013**, *79*, 5997−6005.
(30) Nett, M.; Ikeda, H.; Moore, B. S. *Nat. Prod. Rep.* **2009**, *26*, 1362−1384.
(31) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation: Norwich, UK, 2000.
(32) Gust, B.; Challis, G. L.; Fowler, K.; Kieser, T.; Chater, K. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1541−1546.