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Targeted Capture and Heterologous Expression of the *Pseudoalteromonas* Alterochromide Gene Cluster in *Escherichia coli* Represents a Promising Natural Product Exploratory Platform

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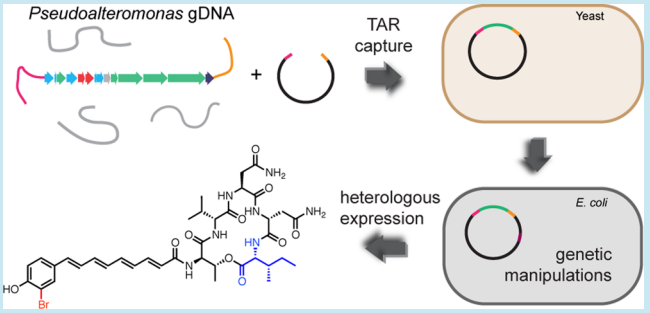
Supporting Information

ABSTRACT: Marine pseudoalteromonads represent a very promising source of biologically important natural product molecules. To access and exploit the full chemical capacity of these cosmopolitan Gram-(−) bacteria, we sought to apply universal synthetic biology tools to capture, refactor, and express biosynthetic gene clusters for the production of complex organic compounds in reliable host organisms. Here, we report a platform for the capture of proteobacterial gene clusters using a transformation-associated recombination (TAR) strategy coupled with direct pathway manipulation and expression in *Escherichia coli*. The ∼34 kb pathway for production of alterochromide lipopeptides by *Pseudoalteromonas piscicida* JCM 20779 was captured and heterologously expressed in *E. coli* utilizing native and *E. coli*-based T7 promoter sequences. Our approach enabled both facile production of the alterochromides and *in vivo* interrogation of gene function associated with alterochromide's unusual brominated lipid side chain. This platform represents a simple but effective strategy for the discovery and biosynthetic characterization of natural products from marine proteobacteria.



KEYWORDS: *heterologous expression, transformation-associated recombination, biosynthesis, marine natural products, lipopeptides*

Microbial natural products represent a valuable chemical reservoir of life-saving medicines and biological probes that have greatly impacted the quality of human health and our fundamental knowledge of the life sciences.[1] Due to high rediscovery rates, traditional activity-guided screening is no longer an effective approach for the discovery of new natural product chemical scaffolds.[2] The immense biosynthetic potential of microbes as revealed by modern genome sequencing efforts, however, confirms the continuing importance of utilizing bacteria as a source of new molecules and scaffolds.[3−5] Indeed, sequencing projects are not only uncovering the extended capacity of well recognized secondary metabolite producers, such as terrestrial actinobacteria[6] but also revealing new bacterial families with substantial encoded biosynthetic capacity.[7] For example, marine actinobacteria have now been recognized as an abundant source of novel bioactive compounds yielding promising anticancer agents such as salinosporamide A (from the genus *Salinispora*),[8] antibiotics such as taromycin (from the genus *Saccharomonospora*),[9] and other drug leads.[10] These observations suggest investigation of the encoded biosynthetic potential of diverse marine bacteria, including proteobacteria, could be very fruitful and ultimately yield unique clinically applicable natural products.

Pseudoalteromonas is a genus of marine γ-proteobacteria found in seawater, marine sediments and epiphytically associated with marine invertebrates. Pseudoalteromonads play a role in biofilm formation and influence settlement, germination and metamorphosis of various marine invertebrate and algal species.[11−14] In recent years, the genus has been identified, through bioactivity chemical profiling, as a promising source of chemically diverse molecules, including antifouling, antibacterial, antifungal, and cytotoxic agents.[15,16] The most thoroughly studied *Pseudoalteromonas* metabolite is thiomarinol A, a very promising antibiotic with broad spectrum activity. Sequencing of the responsible gene cluster has revealed a large hybrid pathway containing fatty acid, polyketide, and nonribosomal peptide synthesis genes.[17]

In light of the genome sequencing results observed for other microbes, it is very likely that the number of pathways encoded within *Pseudoalteromonas* genomes will dwarf the number of molecules identified to date. To probe the chemical capacity of this promising genus, we undertook an analysis of 47 publically available *Pseudoalteromonas* genome sequences using the antiSMASH web-based bioinformatics tool for predicting

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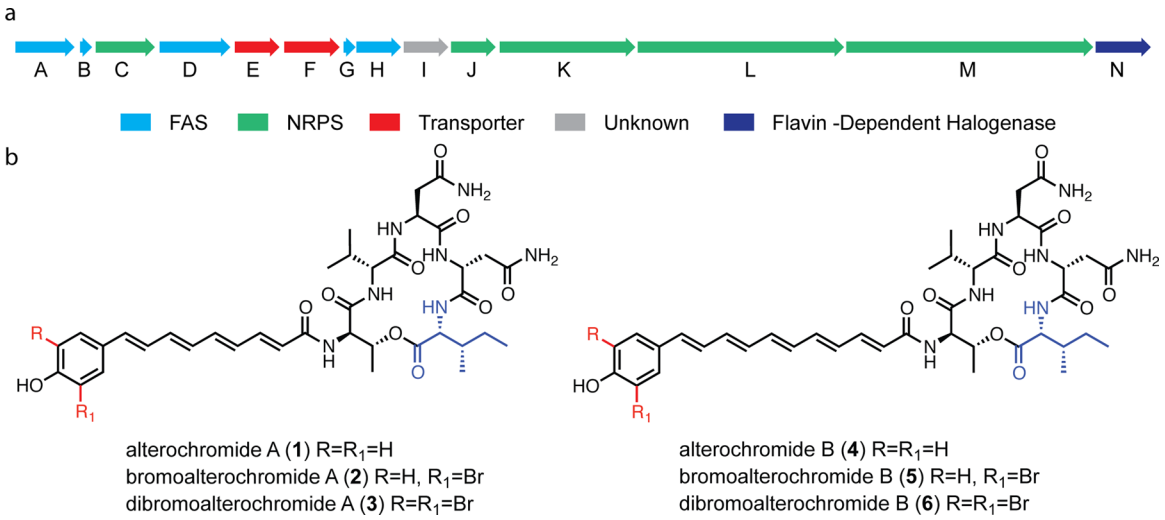

Figure 1. (a) Biosynthetic gene cluster for the alterochomide family of natural products in *Pseudoalteromonas piscicida* JCM 20779.[22] (b) Structures of alterochromide A-like molecules (**1**–**3**) and the alterochromide B-like molecules (**4**–**6**) isolated from *Pseudoalteromonas sp.* differ in the length of the lipid chain and display variable halogenation patterns highlighted in red. Note six further analogues are simultaneously produced in which the D-alloisoleucine residue shown in blue is replaced by D-leucine. These molecules are denoted as alterochromide A′ (**7**), bromoalterochromide A′ (**8**), etc., and dibromoalterochromide B′ (**12**). Finally, the D-alloisoleucine residue shown in blue is also replaced by valine in molecules named following the established nomenclature alterochromide A″ (**13**), bromoalterochromide A″ (**14**), alterochromide B″ (**15**), and bromoalterochromide B″ (**16**). See Supporting Information Figure S4 for a complete display of all structures 1–16. Abbreviations: FAS, fatty acid synthase; NRPS, nonribosomal peptide synthetase.

secondary metabolite pathways.[18] We found that this genus encodes biosynthetic genes for many different classes of molecules, including peptides of both ribosomal and nonribosomal origin, indole derivatives, siderophores, polyketides, homoserine lactones, and hybrid molecules (see Supporting Information Table S1). Representatives of many of these chemical classes have been characterized,[15] as have diketopiperazines[19] and polybrominated pyrroles and phenols[20] that arise from biosynthetic pathways challenging to predict using current algorithms. The most prolific *Pseudoalteromonas* strains contain upward of 20 biosynthetic gene clusters; however, few strains have more than a couple of characterized metabolites, indicating that the chemical bounty of the genus largely remains to be uncovered. To fully realize the considerable biosynthetic potential of these microorganisms, genetic and synthetic biology tools for manipulation and reactivation of their genetically encoded biosynthetic pathways in a host organism must be developed.

Traditional approaches for the capture and manipulation of natural product biosynthetic gene clusters utilize large, randomized genomic libraries and therefore require extensive screening to identify positive clones. Biosynthetic pathways can reach 100 kb in size; however, because cosmid and fosmid library inserts are restricted to around 40 kb, large pathways cannot be captured in their entirety as a single clone and further manipulations are frequently necessary to assemble a complete gene cluster from multiple cosmids.[21] Transformation-associated recombination (TAR) is an alternative, PCR-independent method that utilizes homologous recombination in yeast (*Saccharomyces cerevisiae*) for the direct capture of specific genomic DNA fragments in a selective manner.[22] A specific TAR capture/expression vector can be assembled with sequence information for the target region of genomic DNA, facilitating the *in vivo* interrogation of function for captured genes. In a previous publication, we reported a TAR-based approach in which a silent 67-kb biosynthetic pathway was captured from the genomic DNA of a marine actinomycete and genetically activated, thus facilitating heterologous expression and the discovery of the novel antibiotic taromycin A.[9] TAR represents a paradigm shift in the study of microbial natural products, enabling straightforward and specific capture of target biosynthetic pathways, without the size limitations or inconveniences associated with generating and screening genomic libraries.

Herein, we expand our current TAR capability to include the facile capture of pseudoalteromonad genomic loci utilizing the versatile host organism *Escherichia coli*[21,23] to enable genetic manipulation and direct heterologous expression of pathways in a simple and expedited manner. *E. coli* was chosen because it has a similar GC content, is fast growing, has extensive tools available for its genetic manipulation, and has a limited secondary metabolite profile, generating a "clean" background for gene expression.

We chose to capture and express the putative biosynthetic pathway for a group of lipopeptides exemplified by bromoalterochromide A (**2**) (Figure 1).[24−27] Produced by multiple species of *Pseudoalteromonas* bacteria, including *P. piscicida* JCM 20779, the "alterochromides" possess unusual chemical structural features, such as a brominated lipid moiety, in addition to their antibacterial and cytotoxic properties. Structures of the different peptides within the alterochromide family vary by the length of the lipid moiety (C-15 or C-17), identity of the amino acid at position 5 (isoleucine, leucine, or valine), and by degree of halogenation on the phenolic residue (zero, one or two bromine atoms). The 14 open reading frames (ORFs) of the 34-kb alterochromide (*alt*) gene locus are all aligned in a single direction, and *altB-F* and *altG-M* form overlapping regions encompassing 6 kb and 25 kb, respectively. The gene cluster encodes nonribosomal peptide synthetase (NRPS), fatty acid synthase (FAS), flavin-dependent halogenase,[20] and transporter proteins.

The TAR-based capture of the alterochromide gene cluster directly from *P. piscicida* gDNA is summarized in Scheme 1. Briefly, 1-kb regions located outside the boundary of the gene

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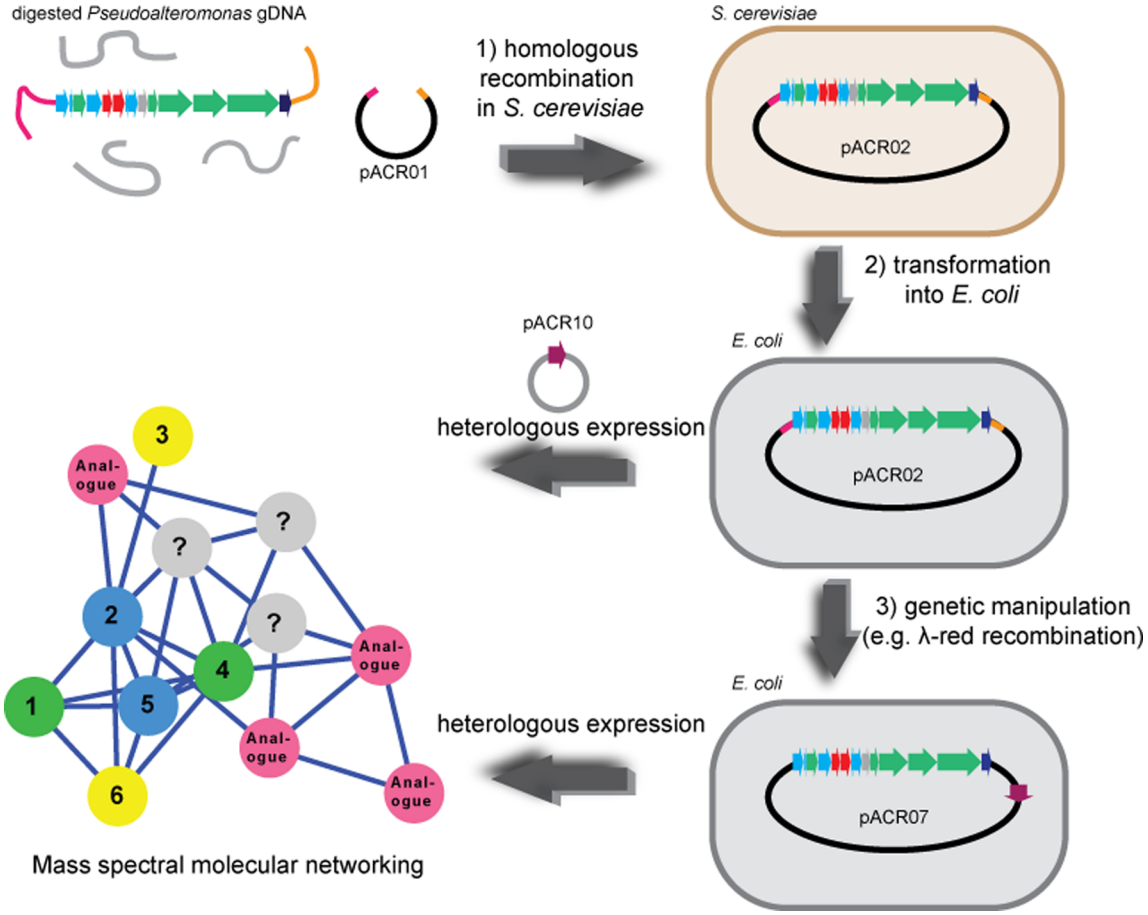
Scheme 1. General Strategy for the Capture and Expression of Biosynthetic Genomic Loci from *Pseudoalteromonas* Exemplified by the Alterochromide Pathway[a]



[a]Step one utilizes a TAR cloning approach of homologous recombination in yeast between enzymatically digested genomic DNA and a pathway specific capture vector. Step two introduces the pathway of interest held within the capture vector into *E. coli*. The expression of the pathway can be attempted directly, perhaps with the assistance of auxiliary genes maintained in a complementary plasmid or integrated into the chromosome of *E. coli*. Step three involves additional genetic manipulation of the pathway in *E. coli* before expression is attempted. Mass spectral molecular networking can be used for comparison of metabolites from the expression systems versus the wild-type producer and is extremely useful for identifying new compounds in addition to confirming production of known molecules.

cluster were amplified, assembled, and cloned into the TAR vector pCAP01[9] to generate the alterochromide specific capture vector pACR01. *Saccharomyces cerevisiae* VL6−48 spheroplasts were transformed with linearized pACR01 and enzyme-digested *P. piscicida* JCM 20779 gDNA. Positive clones containing the captured *alt* gene cluster were identified and confirmed to give pACR02.

With the full biosynthetic pathway for the alterochromide molecules captured, direct expression from the TAR vector was attempted in *E. coli* using the native promoters associated with the *alt* locus. The 34-kb *alt* locus is, to the best of our knowledge, larger than any other *Pseudoalteromonas*-derived or NRPS-containing pathway that has been successfully expressed as a single construct in *E. coli*.[28−32] As the last gene in the 14-ORF *alt* biosynthetic gene cluster encodes a halogenase (*altN*), complete transcription of the pathway should result in heterologous production of the halogenated molecule bromoalterochromide A (**2**), in addition to the des-halo **1**.

Thiolation domains within an NRPS must be functionalized with a phosphopantetheinyl carrier arm;[33] however, no phosphopantetheinyl transferase (PPTase) gene, responsible for this modification, was identified in the neighborhood of the *alt* gene cluster. To ensure effective production of the alterochromide molecules in the *E. coli* heterologous host, we utilized a functionally established PPTase from the related strain *Pseudoalteromonas luteoviolacea* 2ta16.[20,34] The gene-encoded PPTase was cloned into pACYCDuet-1 to generate pACR10, and coexpression with pACR02 was undertaken in *E. coli* BL21 (DE3) using media supplemented with KBr.

Upon analysis of organic culture-extracts by liquid chromatography coupled mass spectrometry (LCMS) (Figure 2a) and metabolic comparison by MS networking[24,35] against natural alterochromides from *P. piscicida* (Supporting Information Figures S1 and S2), we confirmed production of bromoalterochromide A (**2**) as the major heterologously expressed alterochromide. Analysis of the MS data further identified alterochromides **4** and **16**. Trace amounts of alterochromide **4** were detected when pACR02 was expressed in the absence of pACR10 and the auxiliary PPTase. These results confirmed the successful reconstitution of the full alterochromide biosynthetic pathway in *E. coli* using the native *Pseudoalteromonas* promoter sequences.

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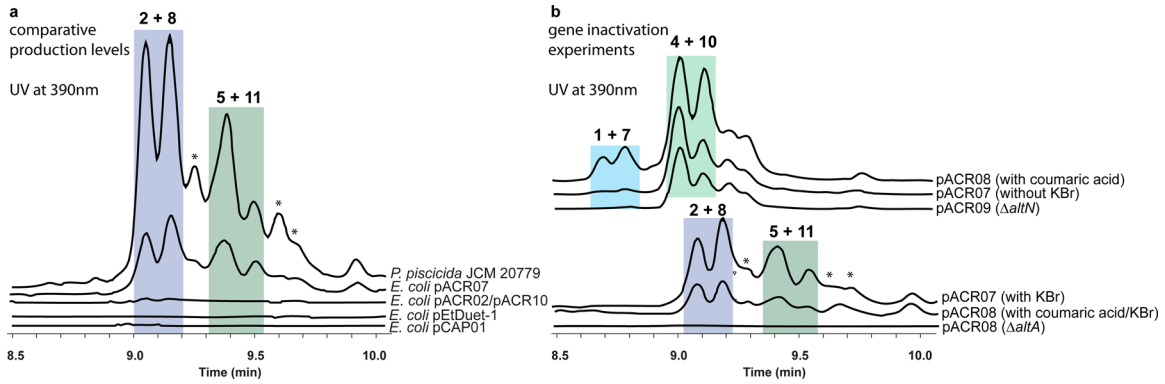

Figure 2. (a) LCMS UV trace showing comparative production levels of alterochromide molecules in the native producer *P. piscicida* JCM 20779 and two *E. coli* heterologous expression constructs (pACR07 and pACR02/pACR10) alongside empty vectors. Bromoalterochromide A (**2**)/bromoalterochromide A′ (**8**) are detected in all three, albeit at levels 3-fold and 60-fold less than the native producer for the expression in *E. coli* with pACR07 and pACR02/pACR10 respectively. (b) LCMS UV trace showing alterochromide metabolite profiles for gene inactivation mutants of alterochromide pathway (Δ*altA* or Δ*altN*) and feeding experiments (Δ*altA*/*coumaric acid*, Δ*altA*/*coumaric acid*/KBr, pACR07/KBr, or pACR07/without KBr), relative production levels not to scale. Note: peaks labeled with an asterisk are other alterochromide compounds.

While we are able to heterologously produce several alterochromide molecules using pACR02, the level of production compared to the *Pseudoalteromonas* native producer was greatly reduced by over 60-fold (Figure 2a). In an attempt to boost production in *E. coli*, we chose to incorporate a T7 promoter directly in front of the 14-ORF *alt* pathway. Several other *Pseudoalteromonas*[28,29] and NRPS biosynthetic pathways have been heterologously expressed in *E. coli* previously under the control of a single non-native inducible promoter (T7, P_{lac_CTU}, or P_{tetO}), with larger pathways spread across multiple expression vectors.[30−32] Although *Pseudoalteromonas* promoters are poorly recognized due to a lack of current information, inspection of the *alt* gene cluster suggested it may potentially be transcribed as a single operon since all genes are oriented in the same direction and with considerable overlap. This organization suggested a promoter exchange in front of the pathway could have a powerful effect on the production levels of the alterochromide molecules as seen in the case of violacein and 3-formyl tyrosine metabolites.[28,29] We applied a λ-red recombination strategy to transfer the full *alt* gene cluster from pACR02 into the first cloning site of the commercial vector pETDuet-1 and introduced the *P. luteoviolacea* 2ta16 PPTase gene into the second cloning site to generate pACR07 in which both cloning sites are under direct control of an upstream T7 promoter (Figure 3). Expression was undertaken in *E. coli* BL21 (DE3) using growth media supplemented with KBr. To our delight, a large number of alterochromide molecules were detected and in significantly higher amounts than in our previous *E. coli* expression, correlating to a 20-fold improvement in production of bromoalterochromide A (**2**) (Figure 2a).

With the successful capture and heterologous expression of the *alt* pathway in *E. coli*, we were provided the opportunity to interrogate the biosynthesis of alterochromide's unusual lipid side chain (Scheme 2). Based on the *alt* gene cluster, we propose that the unsaturated fatty acid chain originates from a tyrosine-derived coumaric acid residue by the action of the AltA tyrosine ammonia lyase (TAL).[36] Moreover, the bromine atom in **2** is likely attached to the coumarate-derived residue by the flavin-dependent halogenase AltN in conjunction with a non pathway-specific flavin reductase,[20,37] yet the exact timing of the bromination reaction was not evident from the DNA sequence.

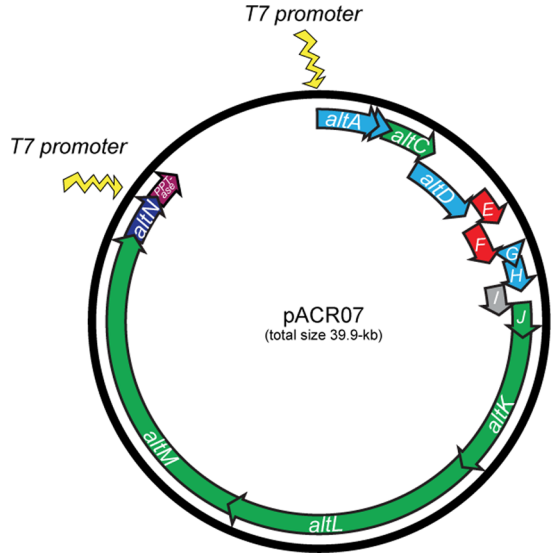

Figure 3. Vector map of pACR07, *altA-N* is arrayed behind a T7 promoter in the first cloning site of pETDuet-1, and the PPTase from *P. luteoviolacea* is contained in the second cloning site.

The *alt* locus also contains genes encoding a nearly complete fatty acid biosynthetic pathway, missing only an enoyl reductase enzyme (FabI), which perfectly correlates with alterochromide's unsaturated lipid chain.

To explore the biosynthetic pathway to the alterochromide lipid, we employed a λ-red recombination approach analogous to that used for the transfer of the full cluster from pACR02 to ultimately create pACR07. Two new pETDuet-1 based expression vectors were generated in which either the TAL-encoding *altA* gene (pACR08) or the brominase-encoding *altN* gene (pACR09) were removed upon transfer. Following heterologous expression in *E. coli* BL21 (DE3), organic extracts from the mutants were similarly analyzed using LCMS and molecular networking (Figure 2b and Supporting Information Figures S2 and S3).

As expected, the *altA* deficient mutant lost the ability to produce alterochromide molecules, which was consistent with the proposed biosynthetic scheme in which the AltA enzymatic product coumaric acid primes the synthesis of the lipid side chain

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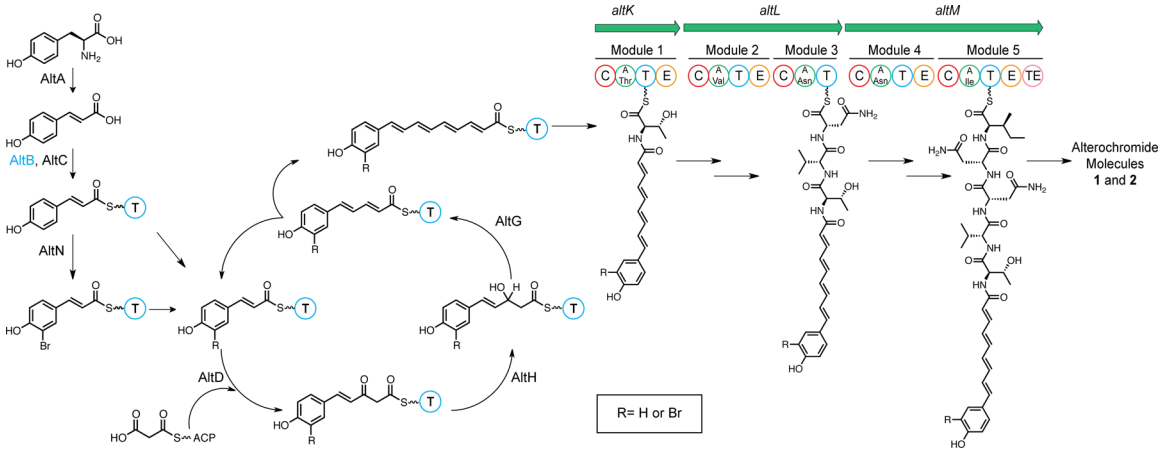
Scheme 2. Proposed Biosynthetic Steps for the Production of the Alterochromide Lipopeptides by the *alt* Gene Cluster in *P. piscicida* JCM 20779[a]



[a]Compounds 1 and 2 are used as an example; however, all molecules in the family are proposed to arise from the same pathway. This can occur through differential halogenation by AltN, passage of the growing lipid through an additional round of fatty acid biosynthesis to give "B" molecules (**4**–**6**) and incorporation of leucine or valine by the 5th NRPS module to give the A′ and B′ (**7**–**12**) and A″ and B″ (**13**–**16**) molecules respectively. Abbreviations: C, condensation domain; A, adenylation domain; T, thiolation domain; E, epimerization domain; TE, thioesterase domain; ACP, acyl carrier protein.

and ultimately the entire molecule. We were able to restore alterochromide production in the *altA* mutant through chemical complementation with supplemental coumaric acid. By contrast, deletion of the halogenase gene *altN* abolished production of just the halogenated molecules, while the nonhalogenated alterochromides **1**, **4**, **7**, and **10** were produced. We observed a similar result with the expression of pACR07 in the absence of supplemental bromide, in which we measured the des-halo alterochromide molecules **1**, **4**, **7**, **10**, and **15**, along with trace amounts of bromoalterochromide B (**5**) that may arise from very low levels of bromide in some of the media ingredients. Although chloride was abundant in the medium, we did not observe any chlorinated alterochromide products, suggesting that the AltN halogenase is specific for bromide. *In vivo* efforts to elucidate the biosynthetic mechanisms responsible for producing the alterochromides are ongoing and further *in vitro* experiments are underway to investigate several enzymatic reactions within the biosynthesis of these unusual lipopeptides, including the exact timing of the halogenation reaction.

In conclusion, the development of a TAR-based genetic platform for the interrogation of the encoded biosynthetic potential of the γ-proteobacterium *Pseudoalteromonas* has-enabled the heterologous expression and functional investigation of the gene cluster responsible for production of the alterochromide lipopeptides. This technology builds upon recent advances in the applications of TAR and features a simple and easy to use refactoring/expression approach based in the common host organism *E. coli*. By demonstrating that *E. coli* is suitable for direct expression of *Pseudoalteromonas* genes, this work leads the way for the facile exploration of the chemistry and biochemistry of this promising marine genus. To this end, efforts are actively underway to develop a high throughput TAR capture method to clone and express pathways in a broad range of host organisms.

METHODS

Construction of pACR01 and TAR Capture of Alterochromide Cluster to Generate pACR02. For capture of the genomic region encompassing the alterochromide gene cluster pACR01 was constructed using pCAP01.[9] Two regions of approximately 1 kb each, flanking the cluster, were amplified by PCR using the following primer sets.

TARBFP	5′-TGC ATC **AAC TAG** TAC TAC GAT ATC GAC GCG CTT-3′ (***Spe*I**)
TARBRP	5′-TCT CTC **AGG ATC** CGT TTT GGA CAA AGC GCA CAG-3′ (***Bam*HI**)
TAREFP	5′-CCA AAA **CGG ATC** CTG AGA GAA TGT CGC CAA TGC-3′ (***Bam*HI**)
TARERP	5′-CGA GTC **ACT CGA** GGC TAT GGG CAG GGC TTA ATG-3′ (***Xho*I**)

The resultant PCR products were assembled by a second PCR reaction using primers TARBFP and TARBRP. The PCR product was digested with *Spe*I and *Xho*I and then cloned into pCAP01 to yield pACR01. The resultant alterochromide specific capture vector (pACR01) was linearized with *Bam*HI and then used in TAR transformation with enzymatically (*Pme*I/*Nae*I) digested *P. piscicida* JCM 20779 gDNA, following a detailed procedure described previously,[9] to generate pACR02 containing the full cluster.

Construction of pACR03. Vector was assembled from pETDuet-1 and the PCR amplified PPTase from *Pseudoalteromonas luteoviolacea* 2ta16 using primers FP2ta16ppt and RP2ta16ppt.

FP2ta16ppt	5′-CAG **CAT ATG** ACT CAG CAC CTT TCA ATA CC-3′ (***Nde*I**)
RP2ta16ppt	5′-CAG **GGT ACC** GGT ACC TTA ACG GCT-3′ (***Kpn*I**)

After restriction digestion with *Nde*I/*Kpn*I, the PPTase was cloned into MCS2 of the linearized pETDuet-1.

Construction of pACR04, pACR05, and pACR06. Expression and gene inactivation vectors for the alterochomide pathway were constructed from pACR03. For each expression construct, two regions (each approximately 1 kb in size) flanking

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the alterochromide cluster were amplified from *P. piscicida* JCM 20779 gDNA by PCR using the following primer sets.

Full Cluster	
BA-Full-B-1	5′-ATA TAT **TCC ATG G**AC CAG TAC AAC ACA GAA AAG GAA GCA ATTC-3′ (***Nco*I**)
BA-Full-B-2	5′-GAC TAA CGC **ACT AGT** GTG ATG CCC ACA TCT CAG CAT CAAT-3′(***Spe*I**)
BA-Full-E-3	5′-GGG CAT CAC **ACT AGT** GCG TTA GTC GCA CGC AAT TACC-3′(***Spe*I**)
BA-Full-E-4	5′-CGC ACG C**AA GCT** TCA CGC CAT TTC AGG GTT GTTAT-3′(***Hind*III**)
altA Deletion Mutant	
altA-KO-B-1	5′-ATA TAT **TCC ATG G**GT AGC TTA GAA TCA ATA ACA CAA TTT ATT AAA CAA GAC ATC ATT GTA-3′(***Nco*I**)
altA-KO-B-2	5′-CGA CTA ACG C**AC TAG T**TG TCA GCA AAG GTG ACG GCTT-3′(***Spe*I**)
altA-KO-E-3	5′-TTG CTG ACA **ACT AGT** GCG TTA GTC GCA CGC AAT TACC-3′(***Spe*I**)
altA-KO-E-4	5′-CGC ACG C**GG ATC C**TC ACG CCA TTT CAG GGT TGT TATGG-3′(***Bam*HI**)
altN Deletion Mutant	
BA-Full-B-1	5′-ATA TAT **TCC ATG G**AC CAG TAC AAC ACA GAA AAG GAA GCA ATTC-3′(***Nco*I**)
altN-KO-B-2	5′-GCA TTT AGC **ACT AGT** GTG ATG CCC ACA TCT CAG CAT CAAT-3′(***Spe*I**)
altN-KO-E-3	5′-TGG GCA TCA C**AC TAG T**GC TAA ATG CAC ACT CAG GCG ATG-3′(***Spe*I**)
altN-KO-E-4	5′-CGC ACG C**AA GCT T**GG AGC TCC CTT TAA TGC CTC ACTC-3′(***Hind*III**)

The first region for each construct was designed such that the first gene in the cluster was aligned with the T7 promoter region and MCS1 start codon of pACR03. The resultant PCR products were assembled by a second PCR reaction using Primers 1 and 4 from each set. PCR products were digested with *Nco*I and *Hin*dIII (or *Bam*HI) and then cloned into the pACR03 MCS1 to yield pACR04, pACR05, and pACR06.

Lambda Red Transfer of Gene Cluster to Generate pACR07, pACR08, and pACR09 (Whole Cluster and Δ*altA* and Δ*altN*). The resultant alterochromide pathway-specific capture vectors (pACR04, pACR05, and pACR06) were linearized with *Spe*I and then transformed into *E. coli* BW25113 containing pACR02 and pIJ790 to complete λ-red recombination to transfer the gene cluster directly into MCS1.

Construction of pACR10. Assembled from pACYCDuet-1 and the PCR amplified PPTase from *Pseudoalteromonas luteoviolacea* 2ta16 using primers FP2ta16ppt and RP2ta16ppt2. After restriction digestion with *Nde*I/*Xho*I, the PPTase was cloned into MCS2 of the linearized pACYCDuet-1.

FP2ta16ppt	5′-CAG **CAT ATG** ACT CAG CAC CTT TCA ATACC-3′ (***Nde*I**)
RP2ta16ppt2	5′-CAG **CTC GAG** GGT ACC TTA ACG GCT-3′ (***Xho*I**)

Analysis of Alterochromide Production by *P. piscicida* JCM 20779. To analyze the production of alterochromide molecules, *P. piscicida* JCM 20779 was cultivated with shaking at 28 °C for 2 days in Difco 2216 marine broth (50 mL) supplemented with KBr (1 g/L). The entire culture was then extracted with ethyl acetate (3 × 100 mL), the organic layers were combined and dried over anhydrous $MgSO_4$. Solvent was removed *in vacuo* and the residue redissolved in methanol and analyzed by LC-MS/MS as follows. A sample was injected onto a reversed phase C_{18} column (Phenomenex luna, 5 μm, 4.6 × 100 mm), operating on an Agilent 1260 HPLC (with UV monitoring at 390 nm) in tandem to an Agilent 6530 Accurate Mass Q-TOF mass spectrometer (positive mode) with a 0.1% formic acid (A)/0.1% formic acid in acetonitrile (B) solvent system. The HPLC method was (flow rate of 0.7 mL/min): 10% B for 2 min, linear gradient to 100% B over 8 min, hold 100% B for 2 min, and linear gradient back to 10% B over 1 min. All the mass spectrometry data and MS/MS of identified spectra for this work has been uploaded into GNPS (MassIVE ID# MSV000078784).

Note: As much as possible, compounds were protected from light throughout all growth, extraction, and purification steps.

Heterologous Expression of Alterochromides in *E. coli*. All heterologous expression was carried out using *E. coli* BL21(DE3) grown in Luria Broth (LB) with appropriate antibiotics (and with or without KBr 1 g/L) added. Bacteria were grown and extracted under identical conditions (except growth medium) to those used for *P. piscicida* JCM 20779.

■ ASSOCIATED CONTENT

Supporting Information

Supporting tables, figures, and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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