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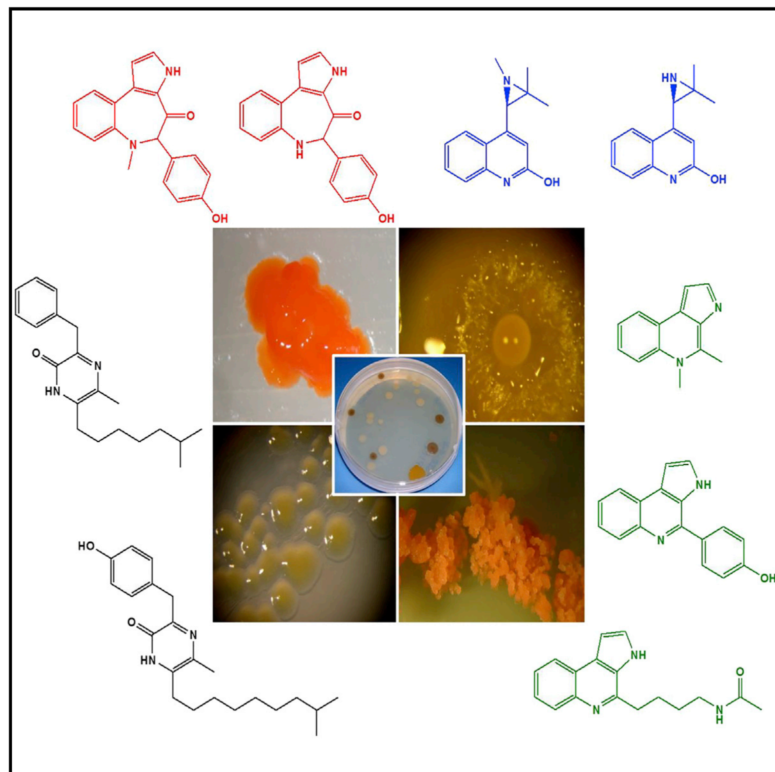
Previously Uncultured Marine Bacteria Linked to Novel Alkaloid Production

Graphical Abstract



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In Brief

Choi et al. illustrate that low-nutrient media and long incubation times lead to the isolation of rare, previously uncultured marine bacteria that produce new antibacterial metabolites. Their work demonstrates that unique marine bacteria are easier to cultivate than previously suggested.

Highlights

- Simple methods allow the isolation of rare, previously uncultured marine bacteria
- Previously uncultured marine bacteria produce new antibacterial metabolites

Accession Numbers

JN703500
JN703501
JN703502
JN703503
JN368460
JN368461
KJ572262
KJ572263
KJ572264
KJ572265
KJ572266
KJ572267
KJ572268
KJ572269
KJ572270
KJ572271
KJ572272
KJ572273
KJ572274
KJ572275

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Previously Uncultured Marine Bacteria Linked to Novel Alkaloid Production

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SUMMARY

Low-nutrient media and long incubation times facilitated the cultivation of 20 taxonomically diverse Gram-negative marine bacteria within the phyla *Bacteroidetes* and *Proteobacteria*. These strains comprise as many as three new families and include members of clades that had only been observed using culture-independent techniques. Chemical studies of the type strains representing two new families within the order *Cytophagales* led to the isolation of nine new alkaloid secondary metabolites that can be grouped into four distinct structure classes, including azepinones, aziridines, quinolones, and pyrazinones. Several of these compounds possess antibacterial properties and appear, on structural grounds, to be produced by amino acid-based biosynthetic pathways. Our results demonstrate that relatively simple cultivation techniques can lead to the isolation of new bacterial taxa that are capable of the production of alkaloid secondary metabolites with antibacterial activities. These findings support continued investment in cultivation techniques as a method for natural product discovery.

INTRODUCTION

Bacteria encompass extraordinary levels of phylogenetic diversity, the vast majority of which has yet to be cultured (Rappé and Giovannoni, 2003). In recent years, however, methodologies involving dilution cultivation (Rappé et al., 2002), the simulation of natural environments (Kaeberlein et al., 2002; Stevenson et al., 2004; Janssen et al., 2002), micro-droplet cell encapsulation (Zengler et al., 2002), and the application of creative sampling devices (Nichols et al., 2010; Aoi et al., 2009; Gavrish et al., 2008; Bollmann et al., 2007), have led to important advances in bacterial cultivation. The application of new techniques such as these is also providing opportunities for natural product discovery, as recently demonstrated by the discovery of the antibiotic teixobactin (Ling et al., 2015). These methods generally avoid the use of traditional, high-nutrient media in favor of more environmentally relevant culture conditions for initial strain isolation. The results make clear that many new groups of bacteria will be available for laboratory-based study if the appropriate cultivation conditions and time course of isolation are employed. Given the historical importance of bacteria as a source of clinically relevant antibiotics (Bérdy, 2005), continued advances in cultivation will likely coincide with increased opportunities for antibiotic discovery.

We previously demonstrated that low-nutrient media improved the cultivation of algae-associated marine bacteria (Jensen et al., 1996). Using a similar approach coupled with extended agar plate incubation periods, we recently cultivated and named two new families of marine bacteria, the *Mooreiaceae* and *Catalimonadaceae*, within the phylum *Bacteroidetes* (Choi et al., 2013). Here, we report the isolation of additional, previously uncultured marine bacterial taxa within the phyla *Bacteroidetes* and *Proteobacteria.* Some of these strains are closely related to bacteria that have only been detected using culture-independent approaches, and thus help to bridge the gap between what can be detected in the environment and what can be studied in the laboratory. Organic extracts of some of these bacterial cultures demonstrated antibiotic activity and led to the isolation of new alkaloidal secondary metabolites, several of which possess new carbon skeletons. These studies further illustrate that new bacterial taxa can be readily isolated using relatively simple cultivation techniques, and that Gram-negative bacteria represent a promising resource for antibiotic discovery, which has traditionally targeted Gram-positive actinomycetes.

RESULTS

Bacterial Cultivation

Using a variety of seawater-based media, we isolated 20 strains of Gram-negative bacteria from various marine sources including sediments, algal surfaces, and invertebrates (Tables S1 and S2). Colonies with yellow to orange pigmentation and a variety of colony morphologies ranging from highly wrinkled to

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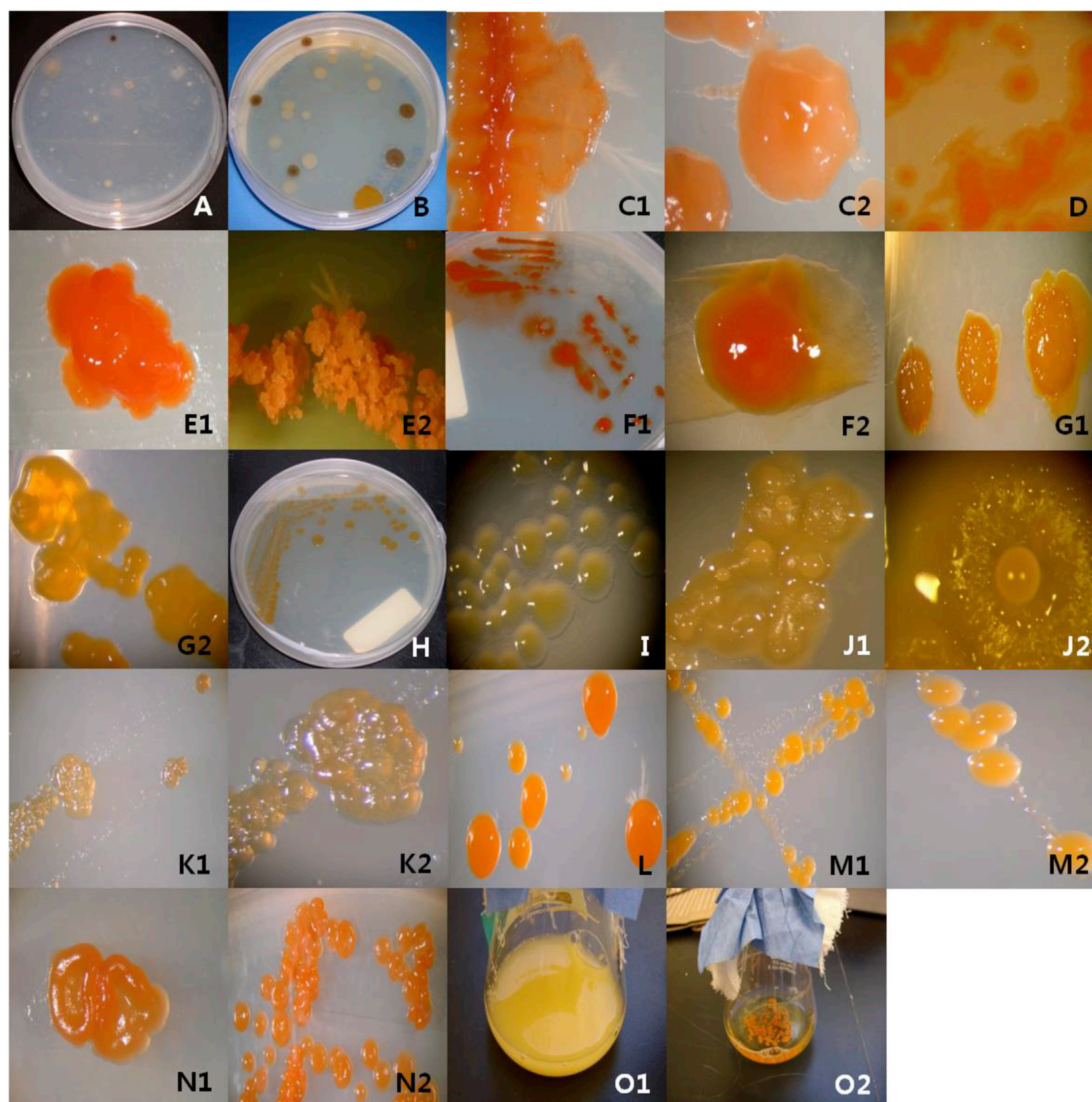

Figure 1. Colony Morphologies of Gram-Negative Strains on Agar Plates and in Liquid Media

(A) Colonies isolated from marine sediment on P1 agar plate. (B) Colonies isolated from marine sediment on P4 agar plate. (C1, C2) Colonies of strain CNU-872 on P1 agar plates. (D) Colonies of strain CNU-893 on P1 agar. (E1, E2) Colony morphologies of strain CNX-216^T on P1 and Nutrient agar plates, respectively. (F1, F2) Colonies of strain CUA-101 on P8 agar plates. (G1, G2) Colonies of strain CUA-116 on P9 agar plates. (H) Isolated strain CUA-117 on P1 agar. (I) Colonies of strain CUA-158 on P10 agar. (J1, J2) Colonies of strain CUA-171 on P9 agar plates. (K1, K2) Colonies of strain CUA-420 on P4 agar plates. (L) Colonies of strain CUA-466 on P7 agar. (M1, M2) Colonies of strain CUA-621 on P7 agar plates. (N1, N2) Colonies of strain TAA-239 on P5 agar plates. (O1, O2) Cultured Gram-negative bacteria in liquid media.

smooth and shiny were targeted (Figure 1). These colonies were generally observed on agar plates following incubation periods of 3 weeks to 2 months. As has been observed in the cultivation of soil- and marine-derived bacteria (Buerger et al., 2012), we also observed that numerous marine colonies were recovered toward the end of the monitoring period, illustrating that extended incubation times, often to several months, can be required for colony formation. Once isolated, all strains grew well in liquid media. Six of these strains were previously reported and two named as type strains for the families *Mooreiaceae* (strain CNX-216^T) and *Catalimonadaceae* (CNU-914^T) as part of a separate study (Choi et al., 2013).

Phylogenetic Analysis and Taxonomic Novelty

A phylogenetic analysis of the 20 strains isolated as part of this study revealed that they are broadly distributed within five orders and two bacterial phyla (*Bacteroidetes* and *Proteobacteria*), with the majority (17 strains) belonging to the *Bacteroidetes* (Figure 2). Based on their phylogenetic relationships and a comparison of 16S rRNA gene sequence identity (SI) with the closest type strains, these cultured bacteria represent candidates for one new family, seven new genera, and one new species in addition to the two new families that have already been described (Choi et al., 2013) (Table 1). The candidate new family shares <86% SI with the nearest type strain and clades exclusively with

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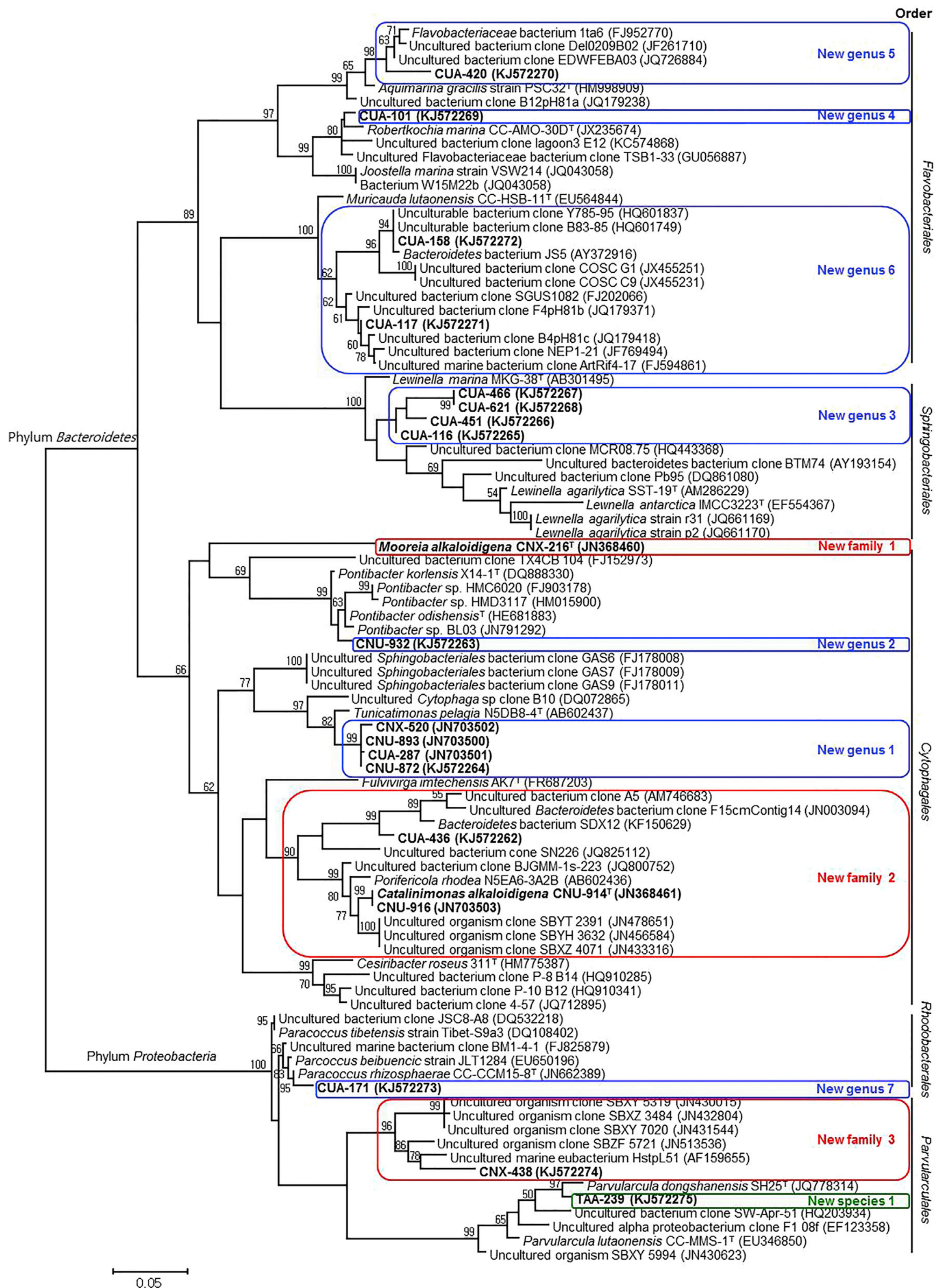

Figure 2. Phylogeny

16S rRNA maximum-likelihood phylogeny for the 20 strains studied here (bold), the most closely related type strains (indicated by T), and the five closest BLAST matches. New and candidate new taxa are indicated. Bootstrap values $\geq$50% (from 1,000 replicates) are shown at their respective nodes.

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Table 1. Bacteria Used in This Study

Number	Strain (Accession Number)	Phylum	Class	Order	Family	Closest Type Strain (Accession Number) (% Identity)	Taxonomic Novelty	Top BLAST Match[a]	
								Culture Dependent	Culture Independent
1	*Mooreia alkaloidigena*[b] CNX-216^{T} (JN368460)	*Bacteroidetes*	*Cytophagia*	*Cytophagales*	*Mooreiaceae*	itself	new family 1	TS[c]	86.0
2	*Catalinimonas alkaloidigena*[b] CNU-914^{T} (JN368461)				*Catalimonadaceae*	itself	new family 2	TS	96.9
3	CNU-916 (JN703503)					*C. alkaloidigena* CNU-914^{T} (JN368461) (99.0)		TS	97.0
4	CUA-436 (KJ572262)					*C. alkaloidigena* CNU-914^{T} (JN368461) (91.6)		TS	94.4
5	CNX-520 (JN703502)				*Flammeovirgaceae*	*Tunicatimonas pelagia* N5DB8-4^{T} (AB602437) (96.3)	new genus 1	TS	90.2
6	CNU-893 (JN703500)					*T. pelagia* N5DB8-4^{T} (AB602437) (93.9)		TS	87.7
7	CUA-287 (JN703501)					*T. pelagia* N5DB8-4^{T} (AB602437) (94.2)		TS	87.9
8	CNU-872 (KJ572264)					*T. pelagia* N5DB8-4^{T} (AB602437) (97.4)		TS	96.7
9	CNU-932 (KJ572263)				*Cytophagaceae*	*Pontibacter odishensis* JC130^{T} (97.0)	new genus 2	TS	94.3
10	CUA-116 (KJ572265)		*Sphingobacteria*	*Sphingobacteriales*	*Saprospiraceae*	*Lewinella marina* MKG-38^{T} (94.9)	new genus 3	TS	94.7
11	CUA-451 (KJ572266)					*L. marina* MKG-38^{T} (95.6)		TS	92.7
12	CUA-466 (KJ572267)					*L. marina* MKG-38^{T} (91.9)		TS	93.5
13	CUA-621 (KJ572268)					*L. marina* MKG-38^{T} (92.9)		TS	93.5
14	CUA-101 (KJ572269)		*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Robertkochia marina* CC-AMO-30D^{T} (97.3)	new genus 4	TS	96.5
15	CUA-420 (KJ572270)					*Aquimarina gracilis* PSC32^{T} (92.8)	new genus 5	94.0	94.9
16	CUA-117 (KJ572271)					*Muricauda lutaonensis* CC-HSB-11^{T} (94.4)	new genus 6	TS	98.8
17	CUA-158 (KJ572272)					*M. lutaonensis* CC-HSB-11^{T} (92.1)		99.4	99.9
18	CUA-171 (KJ572273)	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus rhizosphaerae* CC-CCM15-8^{T} (95.6)	new genus 7	TS	94.6
19	CNX-438 (KJ572274)			*Parvularculales*	*Parvularculaceae*	*Parvularcula dongshanensis* SH25^{T} (85.8)	new family 3	TS	95.2
20	TAA-239 (KJ572275)					*P. dongshanensis* SH25^{T} (98.1)	new species 1	TS	96.9

[a]Not including strains from this study.
[b]From Choi et al., 2013
[c]TS, type strain. Similarity (%) is similarity with closest type strain identity of the left column.

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Table 2. Results of Agar Plate-Assay Disc Antimicrobial Testing for Six Culture Organic Extracts

Organic Extracts	CNJ-912 *Pontibacillus* sp. (mm)	CUA-364 *Vibrio shiloi* (mm)	CNC-294 Undefined Marine Yeast
CNU-916	18	10	not active
CUA-287	17	9	not active
CNU-893	14	not active	not active
CUA-436	12	not active	not active
CUA-116	10	not active	not active
CUA-417	14	not active	not active

sequences that have only been reported using culture-independent techniques, indicating that strain CNX-438 represents the first cultured member of this candidate family. The seven candidate genera all share <98% SI (91.9%–97.4%) with the nearest type strains. Some of these strains, such as CUA-117 (new genus 6), also form diverse clades with sequences that have only been reported using culture-independent techniques. Others, such as those comprising candidate new genus 3, are embedded in clades within previously described genera, in this case *Lewinella* spp. The extraordinary level of diversity encompassed by candidate new genus 3 and the rest of the *Lewinella* clade suggest that this lineage warrants taxonomic revision. The single strain cultured in the family *Mooreiaceae* (CNX-216^T) indicates that this taxon has not been previously cultured or even observed using culture-independent techniques.

Secondary Metabolite Production and Antibacterial Activity

Given the taxonomic novelty of these strains, we next tested their potential to produce novel secondary metabolites with a focus on antibiotic discovery. We screened for activity against the Gram-positive marine bacterium *Pontibacillus* sp. (strain CNJ-912) isolated from marine sediments (Gontang et al., 2007) and the Gram-negative marine bacterium *Vibrio shiloi* (CUA-364), a known coral pathogen (Kushmaro et al., 2001), as well as an undefined marine yeast, our strain CNC-294. We used marine strains for testing based on the concept that marine bacteria-produced antibiotics may preferentially target competing marine bacteria. Organic extracts from five strains (CNU-893, CNX-216^T, CUA-116, CUA-436, and CUA-451) were active against *Pontibacillus* sp. while two strains (CNU-916 and CUA-287) were active against both *V. shiloi* and *Pontibacillus* sp. (Tables 2 and S1). Of these, *Mooreia alkaloidigena* (CNX-216^T) generated some of the more potent antibiotic activities and was thus subjected to detailed chemical analysis, while chemical studies of *Catalinimonas alkaloidigena* (CNU-914^T) were pursued because it was the type strain for the new family *Catalimonadaceae*. None of the extracts inhibited the marine yeast strain. Strains CNX-216^T and CNU-914^T were cultured in replicate 1-l scale, the whole cultures were extracted with ethyl acetate, and a combination of bioassay-guided purification and chemical screening was used to isolate 14 alkaloid secondary metabolites belonging to four structural classes (Figure 3). Of these, compounds **1**–**9** represent new structures, compounds **1**–**4**, **8**, and **9** represent new carbon skeleton structure classes, and compounds **2**, **7**, and **9** represent new antibiotic structure types. The antibiotic activities of the alkaloids isolated are shown in Table 3.

The structure elucidation of the new compounds entailed comprehensive utilization of high-resolution mass spectrometry coupled with one- and two-dimensional nuclear magnetic resonance (NMR) analyses. A complete discussion of the structure elucidations of compounds **1**–**14**, including spectra and detailed discussion of the assignment of relative and absolute configurations, where appropriate, can be found in the supporting information.

Strain CNX-216^T was the more prolific alkaloid producer yielding the new compounds marinoazepinones A and B (**1** and **2**), marinoaziridines A and B (**3** and **4**), and marinoquinolines G–I (**5**–**7**) (Figure 3). Both strains CNX-216^T and CNU-914^T yielded a fourth series of new compounds that we have called the marinopyrazinones A and B (**8** and **9**). Marinoazepinones A and B (**1** and **2**) possess an azepin-3-one moiety. To the best of our knowledge, the marinoazepinones are the first examples of natural products bearing an azepin-3-one functionality. Marinoazepinones A and B also incorporated the unusual, but known, amino acid, 4-hydroxyphenylglycine, which was previously reported in a few natural products, such as the complestatin family (isocomplestatins and chloropeptins). Complestatins are bicylic hexapeptides that possess 4-hydroxyphenylglycine, three modified 4-hydroxyphenylglycines, and tyrosine and tryptophan residues. Complestatins were originally isolated from a *Streptomyces* sp. in 1980 (Kaneko et al., 1989) and were shown to exhibit various biological activities such as inhibition of systemic anaphylactic shock (Kaneko et al., 1980), inhibition of gp120-CD4 binding (Matsuzaki et al., 1994), and inhibition of HIV integrase (Singh et al., 2001).

Marinoaziridines A and B (**3** and **4**) are the first aziridine-containing natural products isolated from Gram-negative bacteria. Other natural products containing an aziridine functionality include mitomycin, ficellomycin, azinomycin, and azicemicin, isolated from Gram-positive bacteria. The azicemicins were originally isolated from the genus of *Amycolatopsis* and were shown to have antimicrobial activity against Gram-positive bacteria (Tsuchida et al., 1993, 1995a, 1995b), while mitomycins (Hata et al., 1956), ficellomycins (Kuo et al., 1989), and azinomycins are produced by Gram-positive bacteria of the genus *Streptomyces* and showed antitumor and antimicrobial activities (Nagaoka et al., 1986; Yokoi et al., 1986). Some aziridine-containing compounds have also been isolated from marine invertebrates (Schaschke, 2004; Salomon et al., 1995).

We also report the isolation of marinoquinolines G–I (**5**–**7**), three new metabolites related to the previously described marinoquinoline A (**10**) (Sangnoi et al., 2008). Marinoquinoline A (**10**) was originally isolated from *Rapidithrix thailandica*, also a member of the phylum *Bacteroidetes*. Marinoquinoline A (**10**) possesses a pyrroloquinoline moiety and has been reported as an acetylcholinesterase inhibitor (Sangnoi et al., 2008). Marinoquinolines B–F (structures not shown) were previously isolated from *Ohtaekwangia korrensis* (Okanya et al., 2011). These compounds showed moderate cytotoxicity to mammalian cell lines and inhibitory activity against the malaria parasite *Plasmodium falciparum* KI. This species is also a member of the phylum *Bacteroidetes* and, like many of the strains reported here, was isolated using low-nutrient media (Yoon et al., 2011). Among

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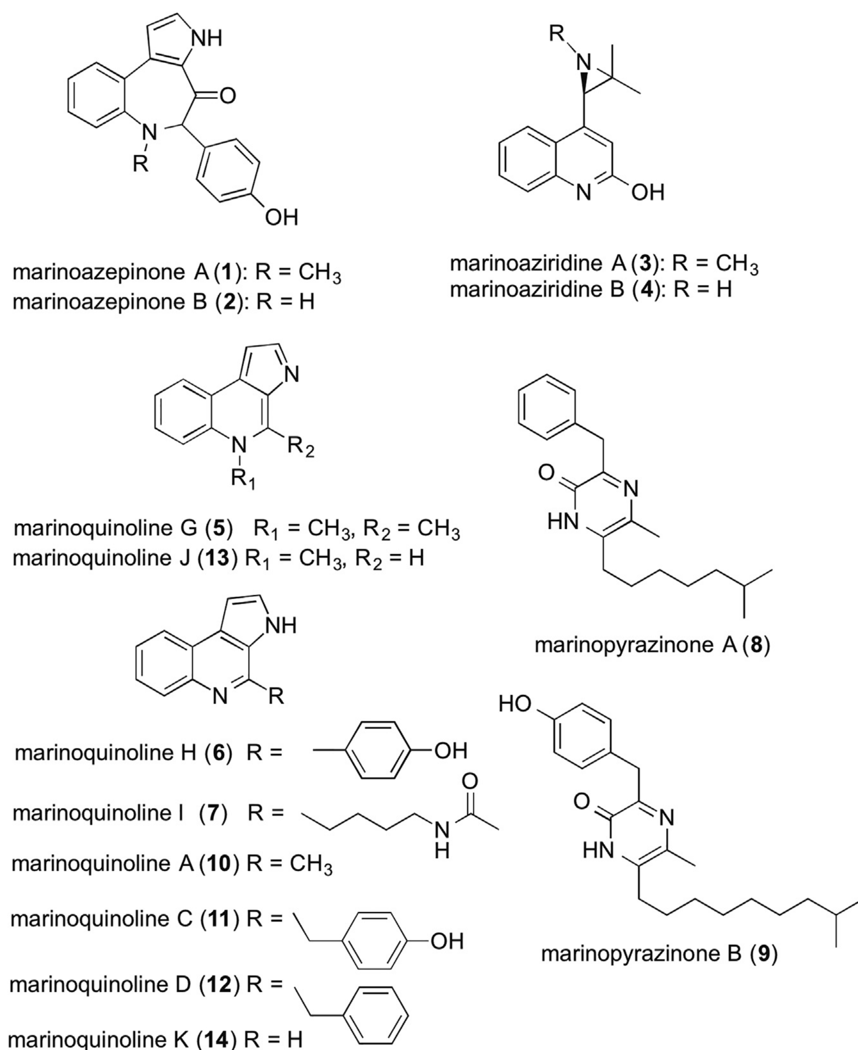

Figure 3. Alkaloidal Metabolites

Chemical structures of secondary metabolites isolated from two strains, *M. alkaloidigena* (CNX-216T) and *C. alkaloidigena* (CNU-914T).

the new marinoquinolines reported here, three alkaloids, compounds **10–12**, displayed antibiotic activity against *Pontibacillus* sp.

Marinopyrazinones A and B (**8** and **9**) are the first pyrazinone natural products isolated from Gram-negative bacteria. Other pyrazinone-containing microbial natural products have been previously reported from the fungus *Aspergillus* sp. and from Gram-positive bacteria of the genus *Streptomyces* (Tatsuta et al., 1971). The compound arglecin, isolated from an *Aspergillus* sp., is the most well-known metabolite because of its antiarrhythmic activity. The compound phevalin, produced by a *Streptomyces* sp., showed inhibitory activity against calpain, a Ca^{2+}-dependent cysteine protease, a promising molecular target for the treatment of neurodegenerative and muscular dystrophy diseases (Alvarez et al., 1995). Recently, two new pyrazinone compounds were isolated from the human pathogen *Staphylococcus aureus* following bioinformatics data analysis (Zimmermann and Fischbach, 2010). The bioinformatics method provided gene sequences that are conserved across all sequenced strains of the human-pathogenic bacterium *S. aureus.* Three pyrazinone secondary metabolites encoded by this nonribosomal peptide gene cluster regulate *S. aureus* virulence factor expression and are essential for the expression of a viable infection in a mouse model (Wyatt et al., 2010).

DISCUSSION

Molecular surveys reveal that the vast majority of bacterial diversity has yet to be obtained in culture (Rappé and Giovannoni, 2003). This gap between what can be observed in the environment and what can be studied in the laboratory severely hampers efforts to exploit bacteria for well-established biomedical purposes such as antibiotic discovery. Using relatively simple methods including low-nutrient agar media and long incubation times, 20 taxonomically diverse strains of Gram-negative bacteria within the phyla *Bacteroidetes* and *Proteobacteria* were cultured. These strains include as many as three new families and seven new genera, indicating that considerable new microbial diversity can be readily cultured with minimal effort. Chemical studies of two of these strains revealed they are a rich source of alkaloid secondary metabolites including new antibiotics and three new structural classes. While it is well known that long incubation times (Stevenson et al., 2004) and low-nutrient media (Connon and Giovannoni, 2002; Janssen et al., 2002) facilitate bacterial culturability, these techniques are generally not applied to the isolation of strains used for antibiotic discovery.

Bacteria in the phylum *Bacteroidetes* are known to be dominant members of soil communities (Fierer et al., 2007) and have been isolated from diverse environments including marine sediments (Choi et al., 2013). While previously recognized for the production of antibiotics (Keller and Zengler, 2004), this phylum and other Gram-negative taxa have traditionally been overlooked in the search for new antibiotics in favor of more traditional microbial resources such as *Streptomyces* spp. Metabolites previously reported from the *Bacteroidetes* include flexirubin and other carotenoids, which are used as chemotaxonomic markers (Reichenbach, 2006; Schmidt et al., 2006), and a series of volatile polysulfides from two *Cytophaga* strains (Sobik et al., 2007). A prior study by our group on the gliding marine bacterium *Saprospira grandis* within the order *Cytophagales* led to the isolation of four new diterpenoids of the neoverrucosane class (Spyere et al., 2003). Similarly, a gliding marine bacterium

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Table 3. Results of Agar Plate-Assay Disc Antimicrobial Testing for Alkaloids 1–14

Pure Compounds **1–14** (50 μg/disc)	Strain CNJ-912 *Pontibacillus* sp. (mm)	Strain CUA-364 *Vibrio shiloi*	Strain CNC-294 Undefined Marine Yeast
Marinoazepinone A (**1**)	not active	not active	not active
Marinoazepinone B (**2**)	16	not active	not active
Marinoaziridine A (**3**)	not active	not active	not active
Marinoaziridine A (**4**)	not active	not active	not active
Marinoquinoline G (**5**)	not active	not active	not active
Marinoquinoline H (**6**)	not active	not active	not active
Marinoquinoline I (**7**)	18	not active	not active
Marinopyrazinone A (**8**)	not active	not active	not active
Marinopyrazinone B (**9**)	14	9	not active
Marinoquinoline A (**10**)	11	not active	not active
Marinoquinoline C (**11**)	16	not active	not active
Marinoquinoline D (**12**)	9	not active	not active
Marinoquinoline K (**13**)	not active	not active	not active
Marinoquinoline J (**14**)	not active	not active	not active
Rifamycin 5 μg	42		
Ciprofloxacin 5 μg		16	

in the genus *Rapidithrix* was the source of the hybrid NRPS-PKS antibiotics ariakemicin A and B (Oku et al., 2008). Among the compounds isolated here are three new derivatives in the marinoquinoline class (**5**–**7**). Related compounds in this series were previously reported from *R. thailandica* and *Ohtaekwangia kribbensis*, suggesting that they may represent a common feature of the order *Cytophagales*.

Biosynthetically, the modification of a variety of amino acid residues can obviously explain the diversity of structures produced by these strains. The side chains in the marinoquinolines and marinoazepinones, for example, can all be hypothesized to be derived from various amino acids. All three of the known marinoquinoline-producing genera belong to the order *Cytophagales* (phylum *Bacteroidetes*), suggesting that this amino acid-based biosynthesis may be a common feature of this order. The origins of several of the alkaloids isolated here are less confidently suggested. Clearly, genomic analysis and annotation would be important as a starting point to assess the biosynthetic origins of the marinoaziridines and marinopyrazinones. The mechanisms of action and molecular targets of the antibacterial alkaloids **2**, **7**, and **8**–**12**, would be of significant interest. Some of these activities may simply be a function of the known antibacterial effects of phenolics, but in other cases this functionality is absent, leading to less confident speculation.

In summary, we report the isolation of 20 taxonomically diverse, Gram-negative bacteria from numerous marine sources including members of as many as three new families within the phylum *Bacteroidetes*. Key to the cultivation of these strains was the use of low-nutrient media and untraditionally long incubation times. Of importance is that two of these strains, the type strains CNX-216^T and CNU-914^T, produce new alkaloid secondary metabolites, several of which represent new carbon skeletons and possess antibacterial activities. Our studies provide support for the use of low-nutrient media and long incubation times in the search for new antibiotics from previously uncultured Gram-negative marine bacteria.

SIGNIFICANCE

This study illustrates that slightly modified but simple cultivation techniques can readily lead to the recovery of bacteria that were previously assigned to the "uncultured majority" including new genus and family level lineages. Chemical studies reveal these strains can be a rich source of new alkaloid secondary metabolites, including some that possess antibiotic activities. The results suggest that many new groups of bacteria can be readily isolated for natural product discovery efforts and that these strains can represent an important resource for antibiotic and other biomedical discovery.

EXPERIMENTAL PROCEDURES

Bacterial Isolation and Cultivation

Marine samples were collected from various locations in sterile Whirl-Pac bags at depths ranging from <1 m to 29 m. Samples were processed as described below (isolation methods 1–7) and in Tables S1 and S2. Plates were checked weekly for emergence of new colonies for the first month and every other week thereafter. Strains were selected for isolation based on pigmentation and morphological features, and obtained in pure culture as previously described (Jensen et al., 2005; Choi et al., 2013).

Method 1

Sediment samples were dried overnight in a laminar flow hood and "stamped" onto the surface of P1, P3, P8, and P11 agar plates using 0.2-cm diameter sterile foam plugs (Jensen et al., 2005).

Method 2

Approximately 1 g of wet sediment was volumetrically added to 4 ml of sterile seawater (SSW), vortex mixed, and heat-shocked at 55°C for 9 min, and 25 μl was inoculated onto P3 agar plates and spread with a sterilized glass rod.

Method 3

Approximately 1 g of wet sediment was volumetrically added to 1 ml of SSW, vortex mixed, and diluted 1:10 in SSW, and 25 μl was inoculated onto P4 agar plates and spread with a sterilized glass rod.

Method 4

Approximately 1 g of algae or soft coral was added to 4 ml of SSW, vortex mixed, and diluted 1:4 with SSW, and 25 μl was inoculated onto P10 agar plates and spread with a sterilized glass rod.

Method 5

Approximately 1 g of sediment or algae was added to 4 ml of SSW, vortex mixed, and heat-shocked at 55°C for 6 min, and 25 μl was inoculated onto P9 agar plates and spread with a sterilized glass rod.

Method 6

Mangrove aerial roots were dried overnight in a laminar flow hood, homogenized with a mortar and pestle, and stamped onto P5 agar plates with a 0.2-cm diameter sterile foam plug.

Method 7

Epidermal mucus from representative elasmobranch species was collected with a sterilized spatula after the epidermal surface was rinsed with SSW for 10 s. Approximately 1 ml of mucus was diluted in 1 ml of SSW, vortex mixed, and further diluted 1:10 in SSW, and 25 μl of each dilution was inoculated onto A1 and P7 agar plates and spread with a sterilized glass rod.

Agar plates were incubated at room temperature (~25°C) for a maximum of 2–3 months and visually inspected weekly for new colonial formation using a Leica MZ6 stereomicroscope (Leica Microsystems). Bacterial colonies that presented features such as dry consistency, irregular margin, and yellow-orange pigmentation were isolated and obtained in pure culture by repeated streaking. Each strain was then cultured in A1 or B1 liquid media (A1: 10.0 g of starch, 4.0 g of yeast extract, 2.0 g of peptone, 750 ml of

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filtered seawater, 250 ml of deionized water; B1: 2.5 g of peptone, 1.5 g of yeast extract, 50% glycerol, and 1 l of seawater) for 7 days while shaking at 230 rpm (25°C) and cryopreserved in 20% (v/v) glycerol at −80°C. In addition, we prepared crude extracts for bioactivity testing by 1-l cultivation in A1 liquid medium (10.0 g of starch, 4.0 g of yeast extract, 2.0 g of peptone, 1 l of filtered seawater) and Difco Marine Broth 2216 for 7 days while shaking at 230 rpm (25°C). The cultures were extracted with the same volume of ethyl acetate, and the ethyl acetate was dried to prepare samples for bioassay.

Bioactive Tests of Gram-Negative Strains

We tested antibacterial activities against *Pontibacillus* sp. (strain CNJ-912) and *V. shiloi* (strain CUA-364), and also an undefined marine yeast, our strain CNC-294, using the agar disc-diffusion bioassay method (Bonev et al., 2008). We added a marine yeast strain to assess eukaryotic activity. *V. shiloi* is a Gram-negative coral pathogen in the family *Vibrionaceae*, while *Pontibacillus* sp. is a Gram-positive marine bacterium belonging to the family *Bacillaceae*. Overnight cultures of these strains were diluted 1:200 in 0.6% molten agar at 44°C (Difco Marine Agar 2216 and A1 agar for *Pontibacillus* sp. and *V. shiloi*, respectively). Organic culture extracts were reconstituted in ethanol at 10 mg/ml, and 25 μl was transferred to a paper disc (250 μg per disc). Fractions were reconstituted at 10 mg/ml in ethanol and 5 μl was transferred to a paper disc (50 μg per disc). After the solvent evaporated, the disc was placed on the agar plate with each test strain. Negative control discs (solvent only) and positive control discs (5 μg of ciprofloxacin BBL Sensi-Disc, Cat. No. 231658) were also placed on test plates.

Phylogenetic Analysis of Gram-Negative Strains

Genomic DNA was obtained from cells derived from agar plates or liquid cultures using the QIAamp DNA Mini Kit (Qiagen). The 16S rRNA gene was PCR amplified using the universal primers 27f and 1492r (Brosius et al., 1978). The PCR products were purified using the MinElute PCR purification kit (Qiagen) and sequenced on a capillary DNA sequencer (Perkin-Elmer model ABI 3730XL, Applied Biosystems) using the ABI Prism Bigdye terminator cycle sequencing Ready reaction kit v.3.1 (Applied Biosystems). The sequences were compared with those available in GenBank using BLAST (Altschul et al., 1990) and the top five matches downloaded. A multiple sequence alignment was generated using Clustal W (Thomson et al., 1994) and a maximum-likelihood phylogeny generated with 1,000 re-sampled datasets (Kimura, 1980; Felsenstein, 1985). A phylogenetic tree was constructed using the program MEGA5 (Tamura et al., 2011).

Fermentation and Extraction

Strain CNX-216^T was cultured at 27°C while shaking at 230 rpm in 80 × 1-l volumes of medium A1BFe + C (composed of 10 g of starch, 4 g of yeast extract, 2 g of peptone, 1 g of $CaCO_3$, 40 mg of $Fe_2(SO_4)_3 \cdot 4H_2O$, 100 mg of KBr, per 1 l of seawater). After 7 days of cultivation, sterilized XAD-16 resin (20 g/l) was added to adsorb extracellular secondary metabolites. The culture and resin were shaken at 215 rpm for 2 hr. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone was removed under reduced pressure, and the resulting aqueous layer extracted with EtOAc (3 × 500 ml). The EtOAc-soluble fraction was dried in vacuo to yield 4.5 g of extract.

Experimental Procedures for Chemical Analysis

The optical rotations were measured using a Rudolph Research Autopol III polarimeter with a 10-cm cell. UV spectra were recorded in a Varian Cary UV-visible spectrophotometer with a path length of 1 cm, and infrared (IR) spectra were recorded on a Perkin-Elmer 1600 Fourier transform IR spectrometer. Circular dichroism (CD) spectra were collected in an AVIV Model 215 CD spectrometer with a 0.5-cm long cell. Proton 1D and 2D NMR spectral data were recorded at 500 or 600 MHz in DMSO-d_6, $CDCl_3$, or methanol-d_4 solution containing Me_4Si as internal standard on Varian Inova spectrometers. ^{13}C NMR spectra were acquired at 75 MHz on a Varian Inova spectrometer. High-resolution electrospray ionization time-of-flight mass spectra were provided by The Scripps Research Institute, La Jolla, CA or the mass spectrometry facility at the Department of Chemistry and Biochemistry at the University of California, San Diego, La Jolla. Low-resolution liquid chromatography-mass spectrometry (LC-MS) data were measured using a Hewlett-Packard series 1100 LC-MS system with a reversed-phase C_{18} column (Phenomenex Luna, 4.6 × 100 mm, 5 μm) at a flow rate of 0.7 ml/min.

Isolation of Marinoazepinones 1, 2, Marinoaziridines 3, 4, Marinoquinolines 5–7 and 10–14, and Marinopyrazinone A 8

The extract (4.5 g) of CNX-216^T was fractionated by open column chromatography on silica gel (25 g), eluted with a step gradient of CH_2Cl_2 and MeOH. The CH_2Cl_2/MeOH 5:1 fraction contained a mixture of both marinoazepinones and marinoaziridines, which were purified by reversed-phase high-performance liquid chromatography (HPLC) (Phenomenex Luna C_{18}(2), 250 × 100 mm, 5 μm, 100 Å, 2.0 ml/min, UV = 210 nm) using an isocratic solvent system (H_2O/CH_3CN = 40:60) to afford marinoazepinones A (**1**, 18.0 mg), B (**2**, 13.0 mg), marinoaziridines A (**3**, 11.2 mg), B (**4**, 13.5 mg), and marinopyrazinone A (**8**, 7.5 mg), as pale yellow oils. The CH_2Cl_2/MeOH 1:1 fraction contained a mixture of marinoquinolines, which was purified by reversed-phase HPLC (Phenomenex Luna C_{18}(2), 250 × 100 mm, 5 μm, 100 Å, 2.0 ml/min, UV = 210 nm) using an isocratic solvent system from 10% to 50% MeOH (0.01% trifluoroacetic acid) over 50 min to afford marinoquinolines G (**5**, 7.0 mg), H (**6**, 8.3 mg), I (**7**, 4.5 mg), A (**10**, 7.1 mg), C (**11**, 3.0 mg), D (**12**,1.5 mg), J (**14**, 5.0 mg), K (**13**, 2.4 mg), as pale yellow oils.

Fermentation and Extraction for Isolation of Marinopyrazinone B 9 from Gram-Negative Bacterial Strain CNU-914^T

Strain CNU-914^T was cultured in 40 × 1-l volumes of the A1BFe + C liquid medium and shaken at 230 rpm at 27°C. After 7 days of cultivation, sterilized XAD-16 resin (20 g/l) was added to absorb the organic products, and the culture and resin were shaken at 215 rpm for 2 hr. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone was removed under reduced pressure, and the resulting aqueous layer was extracted with EtOAc (3 × 400 ml). The EtOAc-soluble fraction was dried in vacuo to yield 3.4 g of extract. The extract (3.4 g) was fractionated by open column chromatography on silica gel (25 g), eluted with a step gradient of CH_2Cl_2 and MeOH. The CH_2Cl_2/MeOH 5:1 fraction was purified by reversed-phase HPLC (Phenomenex Luna C_{18}(2), 250 × 100 mm, 5 μm, 100 Å, 2.0 ml/min, UV = 210 nm) using an isocratic solvent system (H_2O/CH_3CN = 35:65) to afford marinopyrazinone B (**9**, 6.0 mg), as a pale yellow oil.

Physical and Spectral Properties of Alkaloids 1–14

Marinoazepinone A (**1**): pale yellow oil; $[\alpha]_D^{21}$ −1 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.5), 256 (4.3), 318 (3.9), 380 (3.5) nm; IR (KBr) ν_{max} 3,745, 3,230, 1,616, 1,519, 1,251, 1,025, 757 cm^{-1}; ^1H and 2D NMR (600 MHz in DMSO-d_4, 500 MHz in MeOH-d_4), see Table S3; high-resolution electrospray ionization mass spectrometry (HRESIMS) $[M + H]^+$ *m/z* 305.1296 (calcd for $C_{19}H_{17}N_2O_2$, 305.1284).

Marinoazepinone B (**2**): pale yellow oil; $[\alpha]_D^{21}$ −1 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.5), 256 (4.3), 318 (3.9), 380 (3.5) nm; IR (KBr) ν_{max} 3,745, 3,230, 1,616, 1,519, 1,251, 1,025, 757 cm^{-1}; ^1H and 2D NMR (500 MHz, DMSO-d_4, MeOH-d_4), see Table S4; HRESIMS $[M + H]^+$ *m/z* 291.1141 (calcd for $C_{18}H_{15}N_2O_2$, 291.1128).

Marinoaziridine A (**3**): pale yellow oil; $[\alpha]_D^{21}$ −6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 250 (4.3), 282 (3.8), 388 (3.7) nm; IR (KBr) ν_{max} 3,430, 1,670, 1,622, 1,485, 1,341, 942, 771 cm^{-1}; ^1H and 2D NMR (500 MHz, $CDCl_3$, MeOH-d_4), see Table S5; HRESIMS $[M + H]^+$ *m/z* 229.1346 (calcd for $C_{14}H_{17}N_2O$, 229.1335).

Marinoaziridine B (**4**): pale yellow oil; $[\alpha]_D^{21}$ −6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 250 (4.3), 282 (3.8), 388 (3.7) nm; IR (KBr) ν_{max} 3,433, 1,671, 1,624, 1,481, 1,340, 941, 773 cm^{-1}; ^1H and 2D NMR (500 MHz, $CDCl_3$, MeOH-d_4), see Table S6; HRESIMS $[M + H]^+$ *m/z* 215.1185 (calcd for $C_{13}H_{15}N_2O$, 215.1179).

Marinoquinoline G (**5**): pale yellow oil; UV (MeOH) λ_{max} (log ε) 226 (4.6), 240 (4.6), 301 (4.1), 314 (4.0), 327 (3.8) nm; IR (KBr) ν_{max} 3,310, 1,506, 1,217, 770 cm^{-1}; ^1H and 2D NMR (500 MHz, MeOH-d_4), see Table S7; HRESIMS $[M + H]^+$ *m/z* 197.1084 (calcd for $C_{13}H_{13}N_2$, 197.1079).

Marinoquinoline H (**6**): pale yellow oil; UV (MeOH) λ_{max} (log ε) 227 (4.6), 241 (4.6), 303 (4.1), 315 (4.0), 328 (3.8) nm; IR (KBr) ν_{max} 3,312, 1,504, 1,218, 770 cm^{-1}; ^1H and 2D NMR (500 MHz, MeOH-d_4), see Table S8; HRESIMS $[M + H]^+$ *m/z* 261.1034 (calcd for $C_{17}H_{13}N_2O$, 261.1022).

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Marinoquinoline I (**7**): pale yellow oil; UV (MeOH) λ_{max} (log ε) 226 (4.6), 240 (4.6), 301 (4.1), 313 (4.0), 329 (3.8) nm; IR (KBr) ν_{max} 3,312, 1,670, 1,504, 1,218, 770 cm^{-1}; ^{1}H and 2D NMR (500 MHz, MeOH-d_4), see Table S9; HRESIMS [M + H]$^+$ *m/z* 282.1625 (calcd for $C_{17}H_{20}N_3O$, 282.1606).

Marinopyrazinone A (**8**): pale yellow oil; UV (MeOH) λ_{max} (log ε) 226 (4.6), 295 (3.8), 328 (4.4) nm; IR (KBr) ν_{max} 3,025, 2,971, 1,691, 1,621, 1,457, 1,358 cm^{-1}; ^{1}H and 2D NMR (500 MHz, $CDCl_3$, MeOH-d_4), see Table S10; HRESIMS [M + Na]$^+$ *m/z* 335.2093 (calcd for $C_{20}H_{28}N_2ONa$, 335.2094).

Marinopyrazinone B (**9**): pale yellow oil; UV (MeOH) λ_{max} (log ε) 226 (4.6), 294 (3.8), 326 (4.4) nm; IR (KBr) ν_{max} 3,024, 2,970, 1,692, 1,623, 1,455, 1,357 cm^{-1}; ^{1}H and 2D NMR (500 MHz, $CDCl_3$, MeOH-d_4), see Table S11; HRESIMS [M + H]$^+$ *m/z* 357.2536 (calcd for $C_{22}H_{33}N_2O_2$, 357.2537).

Marinoquinoline A (**10**): ^{1}H NMR (500 MHz, MeOH-d_4): δ 8.50 (1H, dd, *J* = 7.8, 1.2 Hz), 8.19 (1H, d, *J* = 2.4 Hz), 8.07 (1H, dd, *J* = 7.8, 1.2 Hz), 7.85 (1H, dd, *J* = 7.8, 7.8 Hz), 7.83 (1H, dd, *J* = 7.8, 7.8 Hz), 7.46 (1H, d, *J* = 2.4 Hz), 3.14 (3H, s); LC-MS [M + H]$^+$ *m/z* 183; [M + Na]$^+$ *m/z* 205. Comparable with literature values, see Okanya et al., 2011.

Marinoquinoline C (**11**): ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.56 (1H, dd, *J* = 7.8, 1.2 Hz), 8.34 (1H, d, *J* = 2.4 Hz), 8.21 (1H, dd, *J* = 7.8, 1.2 Hz), 7.85 (1H, dd, *J* = 7.8, 7.8 Hz), 7.81 (1H, dd, *J* = 7.8, 7.8 Hz), 7.56 (1H, d, *J* = 2.4 Hz), 7.46 (2H, dd, *J* = 7.8, 1.0 Hz), 7.30 (2H, dd, *J* = 7.8, 7.8 Hz), 7.24 (1H, dd, *J* = 7.8, 1.0 Hz), 4.82 (2H, s); see also Table S12. LC-MS [M + H]$^+$ *m/z* 259; [M + Na]$^+$ *m/z* 281.

Marinoquinoline D (**12**): ^{1}H NMR (500 MHz, MeOH-d_4): δ 8.50 (1H, dd, *J* = 7.8, 1.2 Hz), 8.12 (1H, dd, *J* = 7.8, 1.2 Hz), 7.84 (1H, dd, *J* = 7.8, 7.8 Hz), 7.81 (1H, dd, *J* = 7.8, 7.8 Hz), 8.12 (1H, d, *J* = 2.4 Hz),7.46 (1H, d, *J* = 2.4 Hz), 7.17 (2H, d, *J* = 7.8 Hz), 6.75 (2H, d, *J* = 7.8 Hz), 4.67 (2H, s); see also Table S13. LC-MS [M + H]$^+$ *m/z* 275; [M + Na]$^+$ *m/z* 297.

Marinoquinoline K (**13**): ^{1}H NMR (500 MHz, MeOH-d_4): δ 9.33 (1H, s), 8.62 (1H, dd, *J* = 7.8, 1.2 Hz), 8.30 (1H, d, *J* = 2.4 Hz), 8.17 (1H, dd, *J* = 7.8, 1.2 Hz), 7.94 (1H, dd, *J* = 7.8, 7.8 Hz), 7.90 (1H, dd, *J* = 7.8, 7.8 Hz), 7.55 (1H, d, *J* = 2.4 Hz); see also Table S14. LC-MS [M + H]$^+$ *m/z* 169; [M + Na]$^+$ *m/z* 191.

Marinoquinoline J (**14**): ^{1}H NMR (500 MHz, MeOH-d_4): δ 9.47 (1H, s), 8.66 (1H, dd, *J* = 7.8, 1.2 Hz), 8.37 (1H, dd, *J* = 7.8, 1.2 Hz), 8.29 (1H, d, *J* = 2.4 Hz), 8.02 (1H, dd, *J* = 7.8, 7.8 Hz), 7.95 (1H, dd, *J* = 7.8, 7.8 Hz), 7.54 (1H, d, *J* = 2.4 Hz), 4.60 (3H, s); see also Table S15. LC-MS [M + H]$^+$ *m/z* 183; [M + Na]$^+$ *m/z* 205.

ACCESSION NUMBERS

16S rRNA gene sequences reported in this study have been deposited in the GenBank database under accession numbers GenBank: JN703500, JN703501, JN703502, JN703503, JN368460, JN368461, KJ572262, KJ572263, KJ572264, KJ572265, KJ572266, KJ572267, KJ572268, KJ572269, KJ572270, KJ572271, KJ572272, KJ572273, KJ572274, and KJ572275.

SUPPLEMENTAL INFORMATION

Supplemental Information includes a comprehensive discussion of the structure elucidations of alkaloids **1**–**14**, 15 tables, and 60 figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.07.014.

AUTHOR CONTRIBUTIONS

E.J.C. and S.J.N. performed microbial and chemical research contributing equally as first authors of this work. L.P., D.B., and C.A.K. performed the microbial isolations, cultivations, and some of the phylogenetic identifications. P.R.J. and W.F. designed the research, and assisted in implementing and guiding the research. All authors contributed to writing and producing the manuscript.

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