Vanchrobactin and Anguibactin Siderophores Produced by *Vibrio* sp. DS40M4

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The marine bacterium *Vibrio* sp. DS40M4 has been found to produce a new triscatechol amide siderophore, trivanchrobactin (**1**), a related new biscatecholamide compound, divanchrobactin (**2**), and the previously reported siderophores vanchrobactin (**3**) and anguibactin (**4**). Vanchrobactin is comprised of L-serine, D-arginine, and 2,3-dihydroxybenzoic acid, while trivanchrobactin is a linear trimer of vanchrobactin joined by two serine ester linkages. The cyclic trivanchrobactin product was not detected. In addition to siderophore production, extracts of *Vibrio* sp. DS40M4 were screened for biologically active molecules; anguibactin was found to be cytotoxic against the P388 murine leukemia cell line (IC_{50} < 15 μM).

Iron is an essential element required for key biological processes; however, for most microorganisms, obtaining iron is challenging due to the insolubility of iron(III) [K_{sp} of $Fe(OH)_3 = 10^{-39}$] at physiological pH in aerobic environments. Bacteria often require micromolar levels of total iron to prevent iron limitation of growth, yet pathogenic and marine bacteria face similar challenges acquiring iron. The iron concentration in the surface waters of the oceans is very low, at only 0.01−2 nM over most of the world's oceans.[1–3] In mammals, iron is tightly bound by lactoferrin, transferrin, and ferritin, severely limiting the availability of cellular iron.[4] One strategy bacteria use to acquire iron is to produce siderophores, low molecular weight organic chelating compounds that bind iron(III) with high affinity, to solubilize and facilitate iron uptake into the cells.

Two defining characteristics of marine siderophore structures are (1) suites of amphiphilic siderophores composed of different fatty acid appendages attached to a headgroup that coordinates Fe(III)[5–9] and (2) the presence of an α-hydroxycarboxylic acid moiety, such as citric acid or β-hydroxyaspartic acid, which is photoreactive when coordinated to Fe(III).[10–13] Another distinguishing feature of marine siderophores is the predominance of hydroxamic acid or α-hydroxycarboxylic acid moieties as bidentate ligands for Fe(III), whereas relatively fewer marine siderophores have been found to incorporate catechol groups. Exceptions include the catechol-containing alterobactins A and B, pseudoalterobactin, petrobactins, and anguibactin.[12,14–18] Petrobactin, which utilizes a unique 3,4-dihydroxybenzoate moiety, is the siderophore produced by a *Marinobacter* spp. as well as *Bacillus anthracis*, the causative agent of anthrax.[19,20]

In addition to producing petrobactin, *Bacillus* spp. also produce bacillibactin, a triscatechol amide siderophore framed on a cyclic triester backbone of L-threonine (Figure 1).[20] Each threonine amine is appended by glycine that is ligated by 2,3-dihydroxybenzoic acid. Griseobactin, produced by *Streptomyces griseus*, also appears to be a cyclic trimeric ester of 2,3-dihydroxybenzoyl-arginyl-threonine.[21] Enterobactin and the salmochelins are the only other triscatecholamide siderophores reported to date. Enterobactin, isolated from many different enteric and pathogenic bacteria, is a cyclic triester of 2,3-dihydroxybenzoic acid-L-serine. The salmochelins, isolated from *Salmonella enterica* and uropathogenic *E. coli*, are glucosylated derivatives of enterobactin, in which up to two catechols contain glucose at position C-5 (Figure 1).[22–26]

We report herein the isolation and structure determination of a new triscatechol amide siderophore, trivanchrobactin (**1**), a related new biscatecholamide compound, divanchrobactin (**2**), and the known siderophores vanchrobactin (**3**) and anguibactin (**4**) from the marine bacterium *Vibrio* sp. DS40M4. The cyclic trivanchrobactin product was not detected or isolated. In addition the siderophores and extracts of *Vibrio* sp. DS40M4 were screened for biologically active molecules, of which the fraction containing anguibactin was found to be cytotoxic against the P388 murine leukemia cell line.

Results and Discussion

RP-HPLC analysis of the methanol XAD fractions from the culture supernatant of *Vibrio* sp. DS40M4, grown in a low-iron artificial seawater medium, revealed four peaks in the HPLC chromatogram that react with the Fe(III)-CAS complex, consistent with the presence of apo siderophores (see Supporting Information Figure S1).[27] The presence of a catechol group in compounds **1**−**4** was indicated by the positive Arnow assay.[28] High-resolution electrospray ionization mass spectrometry (HRESIMS) established the mass of the molecular ion $[M + H]^+$ of trivanchrobactin (**1**) as *m*/*z* 1156.4626, corresponding to a molecular formula of $C_{48}H_{66}N_{15}O_{19}$; divanchrobactin (**2**) as *m*/*z* 777.3157 with a molecular formula of $C_{32}H_{45}N_{10}O_{13}$; vanchrobactin (**3**) as *m*/*z* 398.1678 with a molecular formula of $C_{16}H_{24}N_5O_7$; and anguibactin (**4**) as *m*/*z* 349.0969 with a molecular formula of $C_{15}H_{17}N_4O_4S$. A comparison of the molecular weights/molecular formulas of **3** and **4** with those of known catechol-containing bacterial siderophores led to the identification of these two compounds as vanchrobactin (**3**)[29,30] and anguibactin (**4**),[31] which was confirmed by additional NMR data (see Supporting Information).

ESIMS/MS analysis of compounds **1**−**3** revealed similar fragmentation patterns. The common ESIMS/MS fragments of **1**−**3** are compared in Table 1. The parent ion mass and the fragmentation pattern observed in the ESIMS/MS of **1** suggest a linear trimer of vanchrobactin ($[M + H]^+$, *m*/*z* 1156.5; $[M + 2H]^{2+}$, *m*/*z* 578.8). The fragmentation of **1** at one serine-ester linkage yields four major peaks, *m*/*z* 777.3 and 759.3, consistent with the mass of two-thirds of **1**, along with *m*/*z* 380.2 and 398.2, consistent with the mass of one-third of **1** (Figure 2). Fragments *m*/*z* 311.2, 262.2, and 137.0, corresponding to the loss of serine, arginine, and the dihydroxybenzoyl moiety from one monomer unit, are also observed in the tandem mass spectrum of **1** (Figure 2 inset). ESIMS/MS of **2** ($[M + H]^+$, *m*/*z* 777.3) reveals two major fragment ions, *m*/*z* 398.2 and

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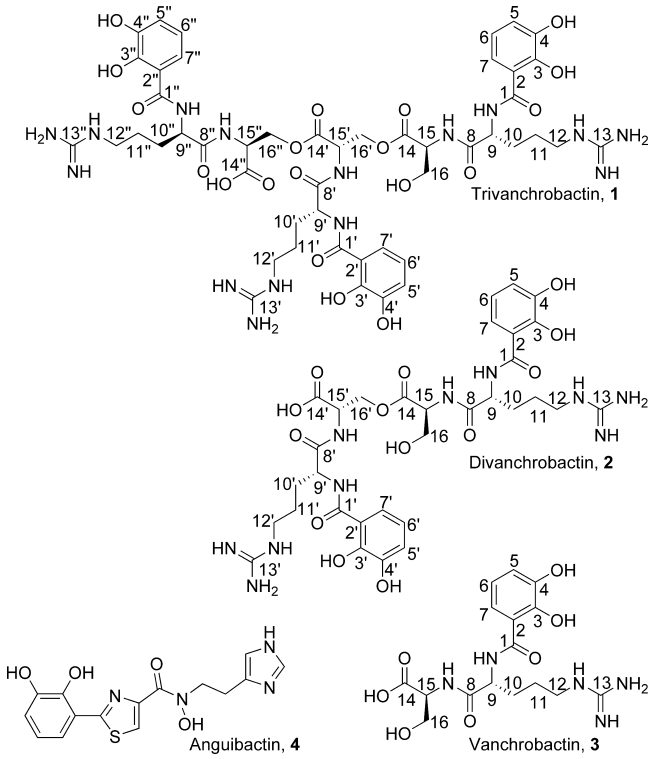

380.2, suggesting a dimer of vanchrobactin, which fragments at the serine ester bond between two vanchrobactin monomer units (Table 1, and Supporting Information Figure S3). Amino acid analysis established the presence of D-arginine and L-serine in **1**−**3**, whereas no amino acids were detected in the hydrolysis products of **4**.

The proton NMR spectra of **1**−**3** are also quite similar, as summarized in Table 2 (and see Supporting Information Figures S4−S12). The aromatic splitting patterns in the ^{1}H NMR spectra of **1**−**3** establish a 2,3-dihydroxybenzoyl moiety. Both the chemical shifts of the serine methylene protons and the integration in **1** (i.e., C16′ δ 4.43, 4.59 and C16″, δ 4.43, 4.69) and **2** (i.e., C16′, δ 4.42, 2H) versus that of vanchrobactin (**3**) indicate the presence of two serine ester linkages in **1** and one serine ester linkage in **2**, thus distinguishing the three related compounds, **1**−**3**, from each other. The methylene protons of the serine hydroxy groups involved in ester formation are shifted downfield relative to the methylene protons of the free serine (i.e., C16) (i.e., δ 3.84, 3.96 for **1**, δ 3.81, 3.93 for **2**, and δ 3.92, 3.96 for **3**). The structure of **1** is confirmed based on ^{1}H−^{13}C HMBC correlations because the methylene protons of the serine residue with the free hydroxy group (C16) are coupled to only one serine carbonyl carbon, whereas the methylene protons of the two serine residues involved in ester formation (C16′, C16″) are each coupled to two serine carbonyl carbons (Figure 5 and Supporting Information Figure S9 and Table S1). The ^{13}C NMR spectrum of **1** has nine distinct carbonyl resonances (δ 169.85 to 174.34), three sp^2 quaternary carbons (δ 158.53, 158.57, and 158.61) corresponding to the arginine guanidine moieties, six methine (δ 53.19 to 54.35), and 12 methylene carbons (δ 26.27 to 41.98 for the arginine residues and δ 62.62, 64.73, and 65.62 for the serine residues). There is some overlap between the aromatic carbon resonances due to the symmetrical character of **1**, and 16 resonances are observed for the 18 aromatic carbons (δ 117.37 to 149.27). The ^{1}H, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC spectra of **3** are consistent with the reported values for vanchrobactin (**3**)[29] and are summarized in Table S2. The structure of **2** was inferred from the tandem mass spectrometry, ^{1}H NMR, and amino acid analyses.

Bioactivity of Anguibactin (4) Produced by *Vibrio* sp. DS40M4. The methanol XAD extract of the supernatant of *Vibrio* sp. DS40M4 was screened for biologically active compounds. Anguibactin was found to be cytotoxic against the P388 murine leukemia cell line (IC_{50} < 15 μM), whereas vanchrobactins **1**−**3** were not cytotoxic.

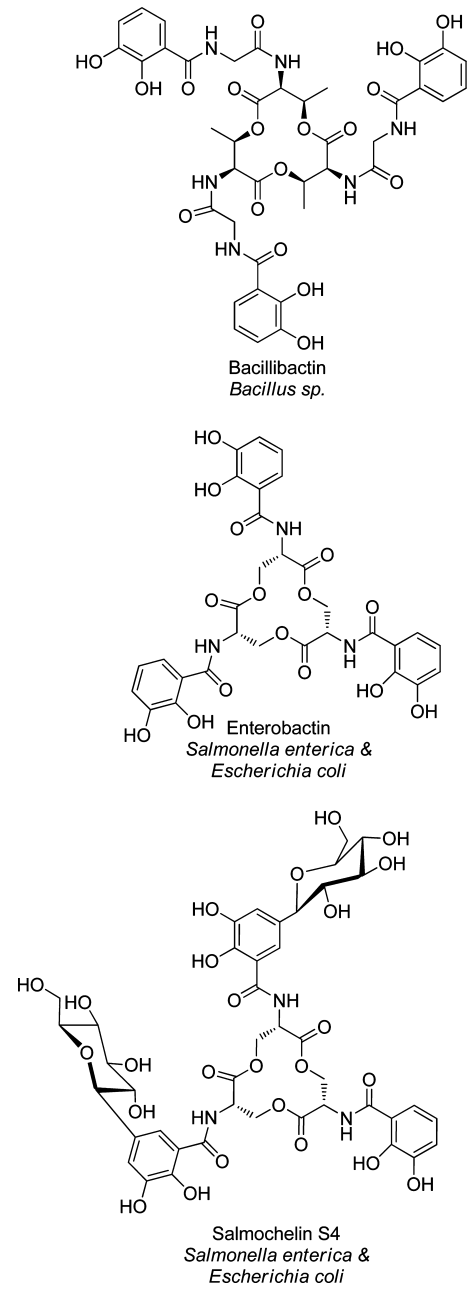

Figure 1. Triscatechol amide siderophores.

Table 1. Molecular Ions and Common Mass Fragments of **1**, **2**, and **3**

trivanchrobactin (**1**) $[M + H]^+$	divanchrobactin (**2**) $[M + H]^+$	vanchrobactin (**3**) $[M + H]^+$
1156.5	777.3	398.2
777.3		
759.3	759.3	
398.2	398.2	
380.2	380.2	380.2
311.2	311.2	311.2
262.2	262.2	262.2

Phylogenetic Analysis of the Bacterial Small Subunit (16S) rRNA Gene. A BLAST search of GenBank using the SSU (16S) rRNA gene from *Vibrio* sp. DS40M4 revealed over 99% similarity to multiple previously described *Vibrio* strains, including *V.*

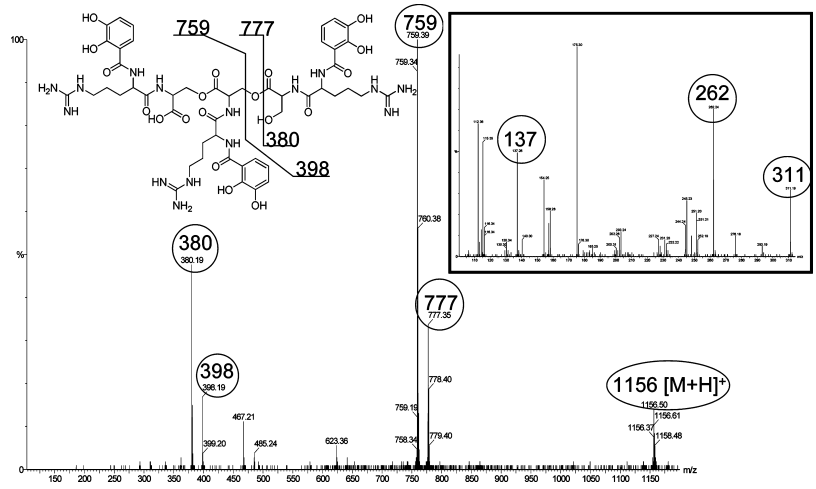

Figure 2. ESIMS/MS of **1**. The inset shows the lower mass range; see structure in Figure S2.

Table 2. Comparison of ^{1}H NMR Chemical Shifts for **1** (800 MHz), **2** (500 MHz), and **3** (500 MHz)

	^{1}H [ppm] *J*(Hz)			
position	**1** (trivanchrobactin) (*Vibrio sp.* DS40M4, CD_3OD)	**2** (divanchrobactin) (*Vibrio sp.* DS40M4, CD_3OD)	**3** (vanchrobactin) (*Vibrio sp.* DS40M4, CD_3OD)	vanchrobactin (*Vibrio anguillarum*, D_2O)[29]
DHBA				
5, 5′, 5″ (CH)	6.96, d (8.0), [3H]	6.95, dd (1.5, 8.0), [2H]	6.96, dd (1.5, 8.0), [1H]	6.83. d (6.9), [1H]
6, 6′, 6″ (CH)	6.76, m, [3H]	6.75, td (1.5, 8), [2H]	6.76, t (8.0), [1H]	6.65, d (6.9), [1H]
7, 7′,7″ (CH)	7.33, d (8.0), [1H] 7.36, d (8.0), [2H]	7.33, dd (1.5, 8.0) [1H] 7.35, dd (1.5, 8.0), [1H]	7.33, dd (1.5, 8.0), [1H]	7.01, d (6.9), [1H]
Arginine				
9 (CH)	4.79, m, [1H]	4.73, dd (3.5, 9.5), [2H]	4.78, dd (5.5, 8.5), [1H]	4.67, dd (4.9, 7.3), [1H]
9′	4.74, m, [1H]			
9″	4.71, m, [1H]			
10, 10′, 10″ (CH_2)	2.03, m, [3H] 1.89, m, [3H]	2.01, m, [2H] 1.84, m, [2H]	2.04, m, [1H] 1.87, m, [1H]	2.33, m, [1H] 2.22, m, [1H]
11,11′,11″ (CH_2)	1.71, m, [6H]	1.70, m, [4H]	1.70, m, [2H]	2.08, m, [2H]
12, 12′, 12″ (CH_2)	3.23, m, [6H]	3.20, m, [4H]	3.23, t (7.0), [2H]	3.41, t (5.9), [2H]
15 (CH)	4.58, t (4.8), [1H]	4.51, t (5.0), [2H]	4.52, t (5.0), [1H]	4.52, t (4.4), [1H]
15′,15″	4.80, d (4.8), [2H]			
16 (CH_2)	3.84, dd (4.0, 11.2), [1H] 3.96, dd (4.8, 11.2), [1H]	3.81, dd (4.5, 11.5), [1H] 3.93, dd (4.5, 11.5), [1H]	3.85, dd (5.0, 11,5), [1H] 3.94, dd (5.5, 11.5), [1H]	4.02 (1H, dd), J = 4.4, 10 3.97 (1H, dd), J = 3.3, 10.5
16′,16″	4.43, m, [2H]			
16′	4.59, m, [1H]	4.42, dd (5.5, 11.5), [2H]		
16″	4.69, m, [1H]			

campbellii, V. rotiferianus, and *V. harveyi*; the relatively conserved SSU rRNA gene has limited power for resolving this closely related group. A phylogenetic tree created by maximum likelihood reveals that *Vibrio* sp. DS40M4 forms a cluster with these strains (Figure 4). *Vibrio* sp. DS40M4 did not, however, cluster with *Vibrio* (*Listonella) anguillarum*, the only other bacterial species known to produce vanchrobactin and anguibactin.

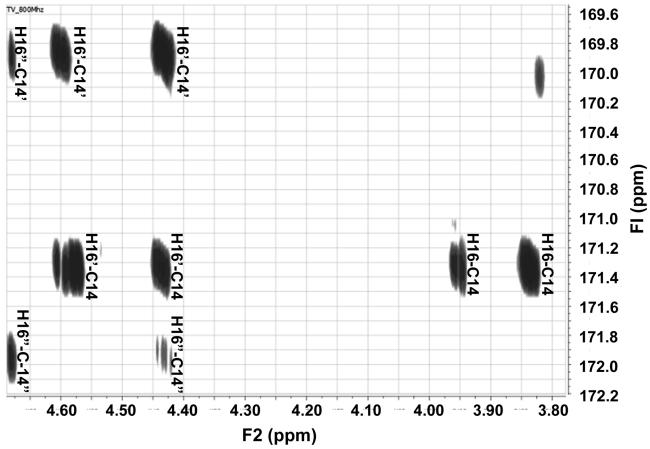

Figure 3. Expanded region of the ^{1}H−^{13}C HMBC spectrum in the vicinity of the serine residues of **1**.

Evaluation of Alternative Phylogenetic Hypotheses. Due to the low bootstrap values supporting the placement of *Vibrio* sp. DS40M4 within the *Vibrio* subgroup shown in Figure 4, alternative hypotheses regarding its phylogenetic placement were tested using the AU test. Constraint trees placing *Vibrio* sp. DS40M4 in neighboring clades were constructed, and the results of that analysis are shown in Table 3. In the original, best scoring likelihood tree, *Vibrio* sp. DS40M4 was placed in clade 1A (Figure 4). A topology placing *Vibrio* sp. DS40M4 in clade 1B, a sister group to clade 1A, did not differ significantly from the most likely tree. However, alternative hypotheses placing *Vibrio* sp. DS40M4 in clade 2, a clade with *V. anguillarum*, were rejected. These results show support for the placement of *Vibrio* sp. DS40M4 within clade 1 and its exclusion from clade 2.

In summary, *Vibrio* sp. DS40M4 produces at least two siderophores, trivanchrobactin and anguibactin, as well as divanchrobactin and vanchrobactin, which may be actual siderophores or simply hydrolysis products of trivanchrobactin. The ESIMS, MS/MS, and NMR analyses presented here establish that **1** is a triscatecholamide siderophore comprised of three vanchrobactin units joined by a diserine ester backbone. The arginine side chain differentiates trivanchrobactin from the other triscatechol amide serine-ester siderophores shown in Figure 1. Vanchrobactin is also structurally similar to the monocatecholamide siderophore chrysobactin produced by the plant pathogen *Erwinia chrysanthemi*. Chrysobactin incorporates a lysine in place of the arginine in vanchrobactin.[32]

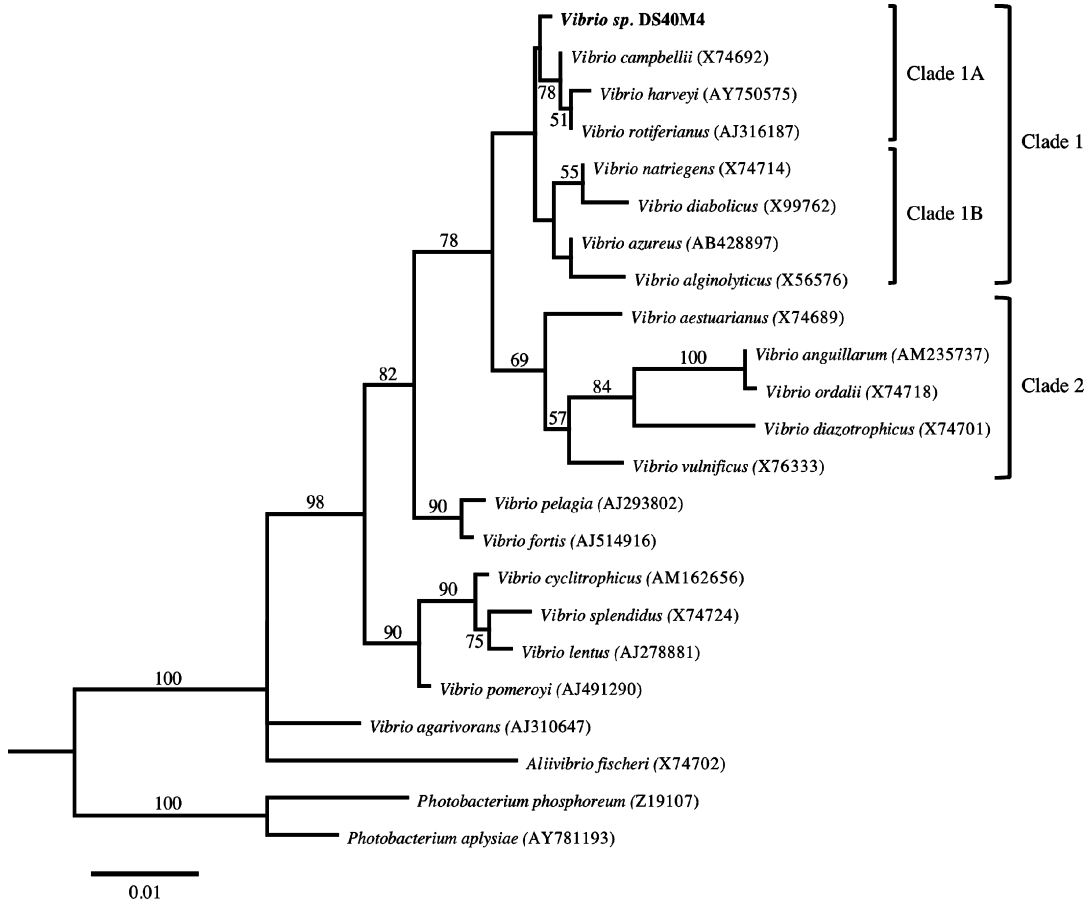

Figure 4. Maximum-likelihood phylogenetic tree showing the placement of *Vibrio* sp. DS40M4 relative to previously described *Vibrio* strains. Bootstrap values over 50% are shown. Scale bar represents 0.01 nucleotide substitution per site.

Table 3. Alternative Phylogenetic Hypotheses with Corresponding log-Likelihoods and *P* Values Inferred from the AU Test[a]

tree	−ln likelihood	difference from best tree	*P* value
best likelihood tree (unconstrained)	3103.685 284	best	
constraint:			
monophyly of DS40M4 with Clade 1B	3107.988 337	4.3030 53	0.269
monophyly DS40M4 with Clade 2	3151.702 524	48.017 24	**0.005**
monophyly of DS40M4, *V. ordalii, V. anguillarum*	3200.726 377	97.0410 93	**<0.001**
monophyly of DS40M4 and *V. anguillarum*	3214.060 006	110.374 722	**<0.001**

[a] Statistically significantly worse trees (rejection of the hypothesis) are those with a *P* value below 0.05 and are shown in bold.

Experiments are underway to explore the potential for other siderophore components produced by *E. chrysanthemi.*

The biosynthesis of vanchrobactin, **3**, is carried out by a previously identified nonribosomal peptide synthetase (NRPS).[33,34] The biosynthesis of trivanchrobactin in *Vibrio* sp. DS40M4 is anticipated to occur similarly, although with two successive iterations of the NRPS system leading to the triserine ester of trivanchrobactin. In the final step of biosynthesis, the covalently bound peptide product must be released from the NRPS. Typically, this reaction is catalyzed by a C-terminal thioesterase (TE) domain. The mechanism by which the TE domain catalyzes product release occurs either via hydrolysis, leading to a linear peptide product, as is expected for trivanchrobactin, or through intramolecular nucleophilic attack, leading to a cyclic peptide product, as occurs for enterobactin,[35] bacillibactin, and the salmochelins.[24,26,36,37] Thus, the thioesterase domain of the EntF component of the NRPS for enterobactin promotes intramolecular attack of the side chain alcohol of the serine on the TE tethered carboxylate of the first loaded serine, releasing the cyclic enterobactin siderophore.[38,39] Experiments are in progress to investigate the mechanism of product release in the biosynthesis of trivanchrobactin, **1**, and in particular to determine whether the linear trivanchrobactin compound is the released product or whether a cyclic triester is formed but not isolated due to the instability of the cyclic triserine ester or the reactivity of a possible esterase. Further experiments will also be directed at identifying the native siderophore; for example is it trivanchrobactin or vanchrobactin, or even divanchrobactin, and does it differ depending on the source bacterium?

Vanchrobactin, **3**, and anguibactin, **4**, are known siderophores produced by various strains of *Vibrio anguillarum*, a fish pathogen causing vibriosis; however they have never before been isolated from the same strain. Vanchrobactin has been isolated from *V. anguillarum* serotype O2 strain RV22,[29] whereas anguibactin has been isolated from *V. anguillarum* serotype O1 strain 775 (pJM1).[31] On the basis of phylogenetic analysis of the SSU rRNA gene, DS40M4 is a *Vibrio* sp. strain that falls within the *Vibrio campbellii* group; it is clearly not a strain of *V. anguillarum* on the basis of the fact that its sequence does not group with that of the *V. anguillarum* type strain (Figure 4). This work expands the distribution of both vanchrobactin and anguibactin to *Vibrio* species other than *V. anguillarum*.

Experimental Section

General Experimental Procedures. ^{1}H, ^{13}C, and 2D NMR (^{1}H−^{1}H gCOSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC) spectra were recorded on Varian INOVA 500 MHz and Bruker Avance II 800 Ultrashield Plus spectrometers in d_4-methanol (CD_3OD; Cambridge Isotope Laboratories). Molecular masses and partial connectivity were determined by electrospray ionization mass spectrometry (ESIMS) and

tandem mass spectrometry (ESIMS/MS), with argon as a collision gas, using a Micromass QTOF-2 mass spectrometer (Waters Corp.). Chiral amino acid analysis was performed using a Varian Saturn 2100T GC-MS fitted with an Alltech Chirasil-Val capillary column.

Bacterial Strain. *Vibrio* sp. DS40M4 was isolated from an open ocean water sample collected over the continental slope off the West Coast of Africa between Cape Verde and the Canary Islands at 20°41.1′N, 24°13.7′W.[40]

Culture and Isolation. *Vibrio* sp. DS40M4 was cultured in low-iron artificial seawater medium (2 L) containing casamino acids (10 g/L), NH_4Cl (19 mM), sodium glycerophosphate hydrate (4.6 mM), $MgSO_4$ (50 mM), $CaCl_2$(10 mM), trace metal grade NaCl (0.3 M), KCl (10 mM), glycerol (41 mM), *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid buffer (10 mM; pH 7.4), $NaHCO_3$ (2 mM), biotin (8.2 μM), niacin (1.6 μM), thiamin (0.33 μM), 4-aminobenzoic acid (1.46 μM), panthothenic acid (0.21 μM), pyridoxine hydrochloride (5 μM), cyanocobalamin (0.07 μM), riboflavin (0.5 μM), and folic acid (0.5 μM) in acid-washed Erlenmeyer flasks (4 L). Four two-liter cultures were grown on an orbital shaker (180 rpm) at room temperature for approximately 24 h until the liquid chrome azurol sulfonate (CAS)[41] test indicated the presence of iron(III)-binding compounds in the culture medium. Cultures were harvested during the stationary phase of growth. After harvesting the cells by centrifugation (6000 rpm, 30 min), Amberlite XAD-2 resin (Supelco) was added to the decanted supernatant (ca. 100 g/L), and the resulting mixture was shaken (7 h at 120 rpm). The XAD resin was washed with doubly deionized H_2O (2 L; Barnstead Nanopure II), and the siderophores were eluted with 100% MeOH. Methanol fractions containing siderophores were identified by the CAS assay and concentrated under vacuum. The siderophores were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a preparative C_4 column (22 mm internal diameter, i.d., × 250 mm length, Vydac) with a gradient from H_2O (doubly deionized with 0.05% trifluoro acetic acid (TFA)) to MeOH (with TFA; 0.05%) over 45 min. The eluent was continuously monitored (215 nm or 210 and 410 nm, simultaneously). Fractions were manually collected and immediately concentrated under vacuum. Samples were ultrapurified chromatographically by preparative C_4 (22 mm i.d. × 250 mm L, Vydac) or semipreparative C_4 column (10 mm i.d. × 250 mm L, Vydac) using the same program as described above. Purified samples were lyophilized and stored at −80 °C.

Siderophores eluted at 24.5 min (**3**), 29.8 min (**2**), 31 min (**1**), and 36 min (**4**). Approximately 0.5−1 mg of vanchrobactins (**1**−**3**) and 8 mg of anguibactin (**4**) were isolated per 4 L of culture.

Trivanchrobactin (1): yellow-brown oil; ^{1}H, ^{13}C, and 2D NMR data, Table 2 and Table S1; HRESIMS *m*/*z* 1156.4626 $[M + H]^+$ (calcd for $C_{48}H_{66}N_{15}O_{19}$, 1156.4659).

Divanchrobactin (2): yellow-brown oil; ^{1}H NMR data, Table 2; HRESIMS *m*/*z* 777.3157 $[M + H]^+$ (calcd for $C_{32}H_{45}N_{10}O_{13}$, 777.3138).

Vanchrobactin (3): yellow-brown oil; ^{1}H, ^{13}C, and 2D NMR data, Table 2 and Table S2; HRESIMS *m*/*z* 398.1678 $[M + H]^+$ (calcd for $C_{16}H_{24}N_5O_7$, 398.1676).

Anguibactin (4): yellow-brown oil; ^{1}H NMR data, Table S3; HRESIMS *m*/*z* 349.0969 $[M + H]^+$ (calcd for $C_{15}H_{17}N_4O_4S$, 349.0971).

Chemical Analysis. Compounds **1**−**4** were tested for the presence of catechol with the Arnow assay,[28] using 2,3-dihydroxybenzoic acid (DHBA) and 3,5-di-*tert*-butyl catechol as standards (~1 mg/mL in H_2O). One milliliter of 0.5 M HCl and nitrite/molybdate reagent (100 g/L $NaNO_2$, 100 g/L $NaMoO_4$) (1 mL) were added sequentially to each sample and mixed well, followed by 0.5 M NaOH (2 mL). The presence of a red color in the solution indicated a positive test for catechol.

Amino Acid Analysis. Marfey's method for amino acid analysis[42] and chiral gas chromatography mass spectrometry (GC-MS) were employed to determine the amino acid composition of the siderophores produced by *Vibrio* sp. DS40M4. For Marfey's analysis,[42] a dry sample of purified siderophore (~1 mg) was first hydrolyzed in hydroiodic acid (55%; ~17 h; 110 °C). The sample was then derivatized using Marfey's reagent (1-fluoro-2,4-dinitrophenyl)-5-L-alaninamide, 1% w/v in acetone) and resolved by HPLC on an analytical YMC QDS-AQ C_{18} column (4.6 mm i.d. × 250 mm L, Waters Corp.) using a linear gradient from triethylamine phosphate (TEAP) (90%; 50 mM; pH 3.0)/CH_3CN (10%) to TEAP (60%; 50 mM; pH 3.0)/CH_3CN (40%) over 45 min. The eluent was continuously monitored on a Waters UV−visible detector (340 nm). Samples were compared to amino acid standards prepared in the same way.

For enantioselective amino acid analysis, a dried, purified siderophore sample (~1 mg) was hydrolyzed with HCl (6 M; ~17 h; 110 °C). The dried sample was derivatized to form the pentafluoropropionyl isopropyl esters of the amino acids and analyzed directly by chiral GC-MS using a Chirasil-Val capillary column, (injection temperature, 220 °C; carrier gas, He (1 mL/min)) using a temperature gradient (80 °C for 3 min, then increased to 200 °C at 5 °C/min). Derivatized samples were compared to amino acid standards prepared in the same way.

Phylogenetic Analysis. Bacterial Small Subunit (16S) rRNA Gene: Amplification and Sequencing. Bacterial primers 27F and 1492R[43] were used to amplify the nearly full-length 16S rRNA gene from genomic DNA. PCR products were analyzed by electrophoresis on an agarose gel (1.2%) to confirm size and specificity. PCR products were cleaned using the QIAquick PCR purification kit (Qiagen) and then directly sequenced. Both strands of the PCR product were fully sequenced using internal primers.[43] Sequencing was carried out on an ABI3700XL at the Molecular and Cellular Biology Core on OHSU's West Campus (Beaverton, OR). Sequences were compiled in Bioedit,[44] resulting in a sequence of 1437 bp in length. The GenBank accession number is HM152762.

Phylogenetic Analysis. The 16S rRNA gene sequence was compared to those in the NCBI nucleotide collection and RDP databases.[45] The 16S rRNA sequence was aligned with the SINA Webaligner (SILVA).[46] Aligned type strain reference sequences were downloaded from SILVA's rRNA database. The multiple sequence alignment was compiled and edited in Bioedit. Hypervariable regions were excluded from the analysis. Phylogenetic reconstruction was performed on unambiguously aligned nucleotide positions with RAxML v. 7.0.4[47] using the General Time Reversible model of nucleotide substitution under the Γ model of rate heterogeneity (GTRGAMMA) with 100 bootstrap replicates. The selected tree topology had the highest likelihood score out of 100 heuristic tree searches, each search beginning with a distinct randomized maximum parsimony starting tree.

Evaluation of Alternative Phylogenetic Hypotheses. Alternative phylogenetic hypotheses for *Vibrio* sp. DS40M4 were evaluated by the approximately unbiased (AU) test.[48] Constraint trees were constructed placing *Vibrio* sp. DS40M4 in various clades appearing in the original unconstrained maximum likelihood tree. RAxML was used with the original data set to infer maximum likelihood phylogenies for each constraint under the GTRGAMMA model using the same parameters as the original search. Site-wise log likelihoods were estimated by RAxML under the GTRGAMMA model for each constraint tree topology. The AU test was implemented by CONSEL v. 0.1j.[49]

Bioactivity Assay. The MeOH XAD extract of the supernatant of *Vibrio* sp. DS40M4 was screened for biologically active compounds following a previously reported procedure.[50] Briefly, extract (50 μg) was separated by analytical RP-HPLC using a linear gradient from H_2O (doubly deionized with 0.05% TFA) to MeOH over 45 min. The eluent was collected into a 96-well microtiter master plate (15 s/well). An aliquot (5 μL) from each well of the master plate was transferred into a daughter plate. The daughter plate was dried under vacuum and each well inoculated with P388 murine leukemia cells and incubated for 3 days. After incubation, the MTT colorimetric assay was used to expose any biologically active compounds present in the plate wells.[51] An HPLC-bioactivity profile was generated by correlating the plate-reader output against well position.

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Supporting Information Available: RP-HPLC of the MeOH XAD-2 extract of the supernatant of *Vibrio* sp. DS40M4, ESIMS/MS of **2** and **3**, ^{1}H NMR spectra (800 Mz) of **1** in CD_3OD, ^{1}H NMR spectra (500 Mz) of **2**, **3**, and **4** in CD_3OD, ^{13}C of **1**, ^{1}H−^{13}C HSQC of **1** and **3**, ^{1}H−^{13}C HMBC of **1** and **3**, ^{1}H−^{1}H TOCSY of **3**, tablulated NMR data for **1** and **3**, and the ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC of the dityrosine-methine-bridged compound isolated from the growth medium. This material is available free of charge via the Internet at http://pubs.acs.org.

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