RESEARCH LETTER

Pseudovibrio denitrificans strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate

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Abstract

Microbial isolate Z143-1 found to be associated with an unidentified tunicate was characterized due to its significant antimicrobial activity. Z143-1 is similar to *Pseudovibrio ascidiaceicola* and *Pseudovibrio denitrificans* in morphological, physiological and biochemical characteristics, except for its ability to ferment glucose and produce a characteristic red pigment. Fatty acid methyl ester analysis revealed a predominance of the fatty acid 18:1 ω7c at 80.55%, at levels slightly lower than the *Pseudovibrio denitrificans* type strain DN34^T (87.7%). The mol% G+C of Z143-1 is 54.02, relatively higher than the *Pseudovibrio denitrificans* type strain DN34^T and *Pseudovibrio ascidiaceicola* with mol% G+C of 51.7 and 51.4, respectively. However, phylogenetic analysis of the 16S rRNA gene sequence of Z143-1 showed 100% similarity with the *Pseudovibrio denitrificans* type strain DN34^T. In this study, the bacterium Z143-1 is reported as a new strain of *Pseudovibrio denitrificans*. While there is no report of a secondary metabolite for *Pseudovibrio denitrificans*, Z143-1 produces the red pigment heptylprodigiosin, also known as 16-methyl-15-heptyl-prodiginine, which shows anti-*Staphylococcus aureus* activity.

Introduction

Marine microorganisms are considered a promising source of novel drugs due to their biodiversity and consequent chemodiversity, which, to date, are largely unexplored. Under varying ecological and physico-chemical conditions in the oceans, microorganisms are able to mutate, evolve and adapt in a particular environment more readily than higher life forms. Adaptation may include the production of specific secondary metabolites that are important in their survival either as free-living organisms in the water or sediment, or in association with other marine organisms (Jensen & Fenical, 1996).

Some marine invertebrates are known to harbor bacterial species of the *Alphaproteobacteria* group (Enticknap *et al.*, 2006; Fukunaga *et al.*, 2006) such as *Roseibium* spp. (Suzuki *et al.*, 2000), *Stappia marina* (formerly *Agrobacterium* species) (Kim *et al.*, 2006), *Roseobacter* (Eilers *et al.*, 2001) and *Pseudovibrio* spp. (Shieh *et al.*, 2004), and some *Alphaproteobacteria* are reported to produce bioactive compounds (Hentschel *et al.*, 2001; Long & Azam, 2001). To date, the genus *Pseudovibrio* has only two reported species: *Pseudovibrio denitrificans* and *Pseudovibrio ascidiaceicola*. In this paper, a bacterium isolated from a Philippine

tunicate was shown by polyphasic taxonomy to be highly similar to *Pseudovibrio denitrificans*. The red pigment it produced was isolated and identified as heptylprodigiosin.

Prodigiosin and its analogs are tripyrrole compounds having activity against Gram-positive bacteria, protozoa, fungi, some mammalian cell lines (Bennett & Bentley, 2000), and also immunosuppressant activity (Han *et al.*, 1997; Kawauchi *et al.*, 1997; D'Alessio *et al.*, 2000). The prodigiosins have been isolated from a variety of microorganisms such as *Serratia marcescens* (Bennett & Bentley, 2000), *Pseudomonas magnesiorubra* (Gandhi *et al.*, 1976), *Vibrio psychroerythreus* (D'Aoust & Gerber, 1974), *Rugamonas rubra* (Austin & Moss, 1986), *Alteromonas rubra* (Gerber & Gauthier, 1979), *Vibrio gazogenes* (Allen *et al.*, 1983), *Pseudoalteromonas denitrificans* (Kawauchi *et al.*, 1997), and also from the actinomycetes *Streptomyces longisporus ruber* (Wasserman *et al.*, 1976), *Streptomyces roseoverticulatus* var. *albosporus* (Gerber, 1975), *Streptomyces hiroshimensis* (Gerber & Lechevalier, 1976), *Streptoverticillium baldaccii* (Brambilla *et al.*, 1995) and *Actinomadura madurae* (Gerber, 1975). Among these sources, *Pseudomonas magnesiorubra, V. psychroerythreus, V. gazogenes, A. rubra* and *Pseudoalteromonas denitrificans*, belonging to the *Gammaproteobacteria* group, were isolated from the marine habitat.

The Z143 isolate described in this paper is the first α-proteobacterium reported to produce heptylprodigiosin or 16-methyl-15-heptyl-prodiginine (MW 351), which had been reported previously in *Pseudomonas magnesiorubra* (Gandhi *et al.*, 1976), *V. psychroerythreus* (D'Aoust & Gerber, 1974), *R. rubra* (Austin & Moss, 1986), and *Methylosinus trichosporum* (Strauss & Berger, 1983), although the nuclear magnetic resonance (NMR) and other spectroscopic data reported in these earlier studies were incomplete and therefore not definitive.

Materials and methods

Sample collection and bacterial isolation

Marine microorganisms were isolated from the outermost and innermost sections of sponges and tunicates, collected by SCUBA from Zamboanga del Norte, Philippines. One gram of each sample was washed with sterile filtered seawater, surface-sterilized with 70% ethanol and rinsed with sterile, filtered seawater. Samples were homogenized using a mortar and pestle and diluted homogenates were streaked onto marine agar (MA, Difco, BD, MD) plates. Among the microbial isolates, Z143-1, isolated from an unidentified tunicate, was noted for its production of a red pigment that demonstrated significant anti-*Staphylococcus aureus* activity in the thin-layer chromatography (TLC) bioautography overlay assay. This strain was deposited in the Natural Sciences Research Institute Culture Collection (University of the Philippines, Diliman, Quezon City, Philippines) as UPCC-1375.

Characterization of Z143-1

The motility test and flagellar staining using basic Fuchsin were performed using established protocols (Leifson, 1951; Krieg & Gerhardt, 1981). Physiological and biochemical tests such as oxidation/fermentation (O/F), nitrate reduction, denitrification and other biochemical tests were also performed using established procedures (Smibert & Krieg, 1981), including rapid test kits such as API20NE and API20E (nonenteric and enteric test kits, Biomerieux Sa) and the BIOLOG GN Microplate™ (Trust Way, Hayward, CA 94545) as prescribed by the manufacturers. Triplicate analyses were performed using all test kits. For BIOLOG, the inoculum was suspended in marine cation supplement (MCS) (Noble & Gow, 1998). For Na^+ requirement, peptone water [peptone (Difco), 10 gL^{-1}] was used supplemented with various concentrations of sodium chloride (0%, 0.5%, 1%, 2%, 3%, 4%, 5%, 7%, and 10%). The cultures were shaken for 4 days at 27 °C and growth was determined on day 4 ($OD_{416\,nm}$, at which the bacterial pigment does not interfere). For determining the optimum temperature for growth, liquid cultures using reconstituted marine broth (MB) were incubated at 27, 37, 40 °C and a refrigeration temperature of 4–10 °C, and the population densities were measured ($OD_{416\,nm}$). Fatty acid methyl ester (FAME) analysis was performed at Microbial ID Inc., Newark, DE.

Genotypic characterization of Z143-1 was carried out by mol% G+C analysis and 16S rRNA gene sequencing and phylogenetic analysis. For mol% G+C analysis, genomic DNA was extracted with phenol and precipitated with isopropanol, as per the modified method from the International Training Workshop on Microbial Identification and Culture Collection Techniques (1999). The hydrolyzed DNA was analyzed by HPLC [ODS type column, Nacalai Tesque cosmosil 5C18, 4.6 mm × 150 mm, eluent 0.02 M $NH_4H_2PO_4$–acetonitrile 20 : 1 (v/v), 1 mL min^{-1}, 270 nm]. Peak areas were calibrated on the basis of a commercially available standard as an equimolar mixture (25% each base).

For 16S rRNA gene analysis, total genomic DNA was extracted using xanthogenate buffer solution (Tillett & Neilan, 1998) and amplified using primers 27Fl (UFP) and 1494Rc (URP). A total reaction volume of 30 μL containing a final concentration of: 1 × buffer, 3 mM $MgCl_2$, 0.3 mM dNTP mix, 10 pmol each of the primers, 0.2 U *Taq* DNA polymerase (Biotech International, Perth, Australia), 1 μL DNA template and milli-Q water to 30 μL. The thermal cycle consisted of an initial denaturation of 94 °C for 4 min, 30 rounds of 94 °C for 10 s, 55 °C for 20 s, 72 °C for 60 s and a final extension of 72 °C for 2 min (Perkin Elmer thermocycler, Perkin Elmer). DNA-amplified products were

purified by ethanol precipitation, which were used for PCR sequencing. Four to five sequencing reactions were performed using the sequencing primers: 27F, 357F, 1224F, 530R, 1100R, 1220R and 1494R (Neilan *et al.*, 1997). The thermal cycle had an initial denaturation of 96 °C for 3 min followed by 30 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The PCR products were ethanol-precipitated and pelleted according to the ABI protocol and sequenced using an ABI system model 377 at the Automated Sequencing Unit, UNSW, Sydney, Australia. The DNA sequences were edited using the ABI computer programs FACTURA, AUTOASSEMBLER and SEQUENCE NAVIGATOR (Bernardo *et al.*, 1998). Evolutionary affiliations were determined by BLAST (Basic Local Alignment Search Tool) provided by NCBI (Altschul *et al.*, 1990) and aligned with the closest neighbors using CLUSTAL W (Thompson *et al.*, 1994). A phylogenetic tree was constructed using the Kitsch algorithm (Fitsch–Margoliash and least squares method with an evolutionary clock) from the PHYLIP program (Felsenstein, 1989). The sequence was deposited in the NCBI GenBank under accession number AY762960.

Isolation and identification of the active metabolite

The bioactive secondary metabolite was isolated according to the following procedure. A single colony of Z143-1 from a 1-day-old MA plate culture was inoculated onto 25 mL MB in a 125 mL Erlenmeyer flask, shaken for 20 h at 27 °C, 130 r.p.m., using a TEQ^{TM} orbital shaker-incubator. Five milliliters from this culture was inoculated into 100 mL MB in a 500 mL Erlenmeyer flask and shaken overnight under the same conditions. From this culture, 25 mL was inoculated into each of two 2.8 L Fernbach flasks with 500 mL MB broth supplemented with 2 gL^{-1} D-Glucose and shaken at 175 r.p.m., 27 °C, for 2 days. The culture broths from the two Fernbach flasks were pooled and an equal volume of methanol was added. To the solution, an equal volume of hexane was added, shaken in a separatory funnel and the resulting two layers were separated. Hexane extraction was performed thrice, and the resulting extract was dried *in vacuo* at 40 °C. ^{1}H-NMR and MS-electron spray ionization (ESI) were performed to monitor the isolation. The sample was redissolved in trifluoroacetic acid (TFA)-methanol (pH 1.1) and extracted with hexane to remove fatty acids, while the methanol extract was neutralized with $NaHCO_3$ to pH 7.2. The methanol extract was chromatographed twice on an S/P® Silica Gel 60 (70–240 mesh) column (24 mm × 960 mm) using a step gradient of 100% hexane to 100% ethyl acetate to 1 : 1 ethyl acetate : methanol (100 mL). The chemical profile and bioactivity of the column fractions were monitored by the TLC bioautographic overlay assay according to the procedure of Hamburger & Cordell (1987) vs. methicillin-susceptible (MSSA, ATCC 12600) and methicillin-resistant *S. aureus* (MRSA, ATCC 33591) using plastic-backed silica gel plates (F_{254}, E. Merck) and 9 : 1 chloroform : methanol as a developing solvent. TLC plates were examined for colored spots and viewed under short UV (254 nm) and long UV (366 nm), while a duplicate chromatogram was tested for nitrogen by spraying with a chlorine-*o*-toluidine reagent after a 30-min exposure to chlorine gas (Krebs *et al.*, 1969). To remove metal contaminants and other impurities that could interfere with spectroscopic analyses, the pooled active fractions were chromatographed through a cationic exchanger (Biorex-70, Bio-Rad), using an acetonitrile : H_2O : TFA step gradient (pH from 8 to 2 in 1 U increments). The TLC-pure isolate was analyzed by MS (EIMS), NMR (^{1}H-NMR, ^{13}C-NMR, DEPT, NOESY, TOCSY, COSY, HMBC and HMQC), UV and infrared spectroscopy.

Structure elucidation of heptylprodigiosin

Infrared spectrum was recorded using a Jasco FTIR-420 spectrophotometer (NaCl disk). NMR spectra were acquired on a Varian instrument, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. All NMR spectra were recorded in $CDCl_3$, using residual $CHCl_3$ as an internal reference. EIMS analyses were performed on a Finnigan MAT95 (70 eV) spectrometer.

Results and discussion

Morphological, physiological and biochemical characteristics of microbial isolate Z143-1

The morphological, physiological and biochemical characteristics of the bacterial isolate Z143-1, *Pseudovibrio denitrificans* type strain DN34^{T} and *Pseudovibrio ascidiaceicola* type strain F423^{T} (Table 1) indicated that the three microorganisms are similar, except for their ability to ferment glucose. Z143-1 produces white translucent colonies on MA when young, which turn red as the culture ages due to a nondiffusible, nonfluorescent red pigment. On the other hand, no secondary metabolites, including pigments, have been reported for the other two microorganisms. A typical colony of this new isolate is smooth, circular, convex, opaque, with an entire margin and butyrous texture. Like *Pseudovibrio denitrificans* and *Pseudovibrio ascidiaceicola*, Z143-1 is a Gram-negative, rod-shaped, motile, polarly flagellated and oxidatively metabolizing chemoorganotrophic organism (Table 1). It tested positive for oxidase, catalase, urease and β-galactosidase, and negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, agarase, citrate utilization, H_2S production and acetoin production. Like *Pseudovibrio denitrificans*, it is capable of denitrification, a significant characteristic of

Table 1. Taxonomic Characteristics of isolate Z143-1 and *Pseudovibrio denitrificans* type strain DN34^T and *Pseudovibrio ascidiaceicola* type strain F423^T

Characteristics tested	*Pseudovibrio denitrificans* strain Z143	*Pseudovibrio denitrificans* type strain DN34^{T*}	*Pseudovibrio ascidiaceicola* type strain F423^{T†}
Gram reaction	−	−	−
Cell shape	Rod, straight or curved	Rod, straight or curved	Rod, straight or curved
Motility	+	+	+
Flagellar arrangement	Polar	Lateral or subpolar	Subpolar
Optimum temperature for growth	30 °C	30 °C	Grows at 10–30 °C
Optimum NaCl requirement for growth	3%	3%	3–5%
Fermentation of glucose	−	+	+
NO_2 production	+	+	+
Reduction to N_2 gas	+	+	+
Colony characteristics	Circular, nonluminescent, with an entire margin, translucent when young and turns opaque red on marine agar as the culture ages	Circular, nonluminescent, with an entire margin, translucent	Circular, smooth and brownish green in color
Secondary metabolite production	Heptylprodigiosin	ND	ND
Oxidase	+	+	+
Catalase	+	+	+
DNAse	ND	+	ND
Gelatinase	+	+	+
Lipase	ND	+	ND
Agarase	−	−	ND
Amylase	ND	−	ND
Arginine dihydrolase	−	−	+
Lysine decarboxylase	−	−	ND
Ornithine decarboxylase	−	−	ND
Citrate utilization	−	−	ND
H_2S production	−	−	ND
Urease	+	ND	+
Esculin hydrolysis	+	ND	+
β-Galactosidase	+	ND	ND
β-Glucosidase	ND	ND	+
Indole production	+	ND	+
Acetoin production	−	ND	ND
Mol% G+C	54	51.7	51.2

*Data from Shieh *et al*. 2004.
†Data from Fukunaga *et al*. 2006.

Pseudovibrio denitrificans. It can also hydrolyze gelatin and esculin, metabolize acid from glucose and produce indole. It utilized a total of 53 out of 95 substrates in the BIOLOG GN Microplate^{TM} assay (data not shown). Optimal growth was observed at 30 °C. Z143-1 has an apparent dependence on Na^+ for growth (1–10% NaCl), a distinctive characteristic of marine bacteria. Optimal growth was observed at 3% NaCl. The ten fatty acids detected in Z143-1 via FAME analysis are listed in Table 2. The fatty acid 18 : 1 ω7c was predominant in Z143-1 at a level of 80.55%, whereas in *Pseudovibrio denitrificans*, this fatty acid was present at 87.7% and in *Pseudovibrio ascidiaceicola* at 60.2–87.8% (Fukunaga *et al*., 2006).

Z143-1 has a mol% G+C of 54.02, which is relatively higher than those of *Pseudovibrio denitrificans* type strain DN34^T and *Pseudovibrio ascidiaceicola*, at 51.7 and 51.2, respectively.

Phylogenetic characterization of strain Z143-1

Figure 1 shows the evolutionary affiliations between Z143-1 and related species based on 16S rRNA gene sequence analysis. The 16S rRNA gene sequence of Z143-1 was compared with bacterial sequences available in the GenBank database. To highlight the relationship between Z143-1 and other α-proteobacterial species, *Stappia marina, Crassostrea virginica* symbiont, *Roseibium hamelinensi, Achromobacter* sp., *Ochrobactrum* sp. and *Agrobacterium gelatinovorum* were also included in the tree. Further, other bacteria belonging to the *Gammaproteobacteria* group, specifically

Table 2. Fatty acid methyl ester analysis of isolate Z143-1, *Pseudovibrio denitrificans* and *Pseudovibrio ascidiaceicola*

Fatty acid	*Pseudovibrio denitrificans strain Z143*	*Pseudovibrio denitrificans**	*Pseudovibrio ascidiaceicola**
12:0 3-OH	–	–	–
14:0 2-OH	–	–	–
14:0 3-OH	–	–	–
16:0	9.14	4.0	1.6–1.7
16:0 3-OH	1.21	ND	ND
17:0	–	tr	–
18:1 ω7c	80.55	87.7	60.2–87.8
18:0	tr	1.0	3.5–5.5
18:2 ω9, 12	–	–	–
19:0 cyclo ω8c	1.73	tr	8.2–30.9
18:0 3-OH	1.68	1.4	1.6–3.7
19:0 10 methyl	tr	ND	ND
20:0	–	–	–
Summed feature 2	1.89	1.7	2.5–2.2
Summed feature 3	2.01	1.3	3.8–3.8

*Data from Fukunaga *et al*. 2006.

marine bacteria that produce prodigiosin, such as *Pseudoalteromonas rubra, Pseudoalteromonas denitrificans* and *V. gazogenes*, were likewise included in this phylogeny.

Z143-1 formed a cluster with two *Pseudovibrio* species at 99% and with *Pseudovibrio denitrificans* DN34 at 100%. It also formed a clade with α-proteobacterium MBIC3368 at 99.93%. This particular strain has been recovered from various marine sponges (Scheuermayer *et al*., 2006), its bioactivity reported twice (Hentschel *et al*., 2001; Thiel & Imhoff, 2003), and is by far the only isolate recovered by both cultivation and 16S rRNA gene-based approaches (Thoms *et al*., 2003), making it an excellent model for bacteria–sponge interaction studies (Scheuermayer *et al*., 2006). From a biological standpoint, it is worth mentioning that closely related isolates of this strain were found to be vertically transmitted via sponge larvae (Enticknap *et al*., 2006).

To further determine the degree of relatedness of Z143-1 strain to *Pseudovibrio denitrificans* type strain DN34^T, DNA-based typing of this strain such as pulsed field gel

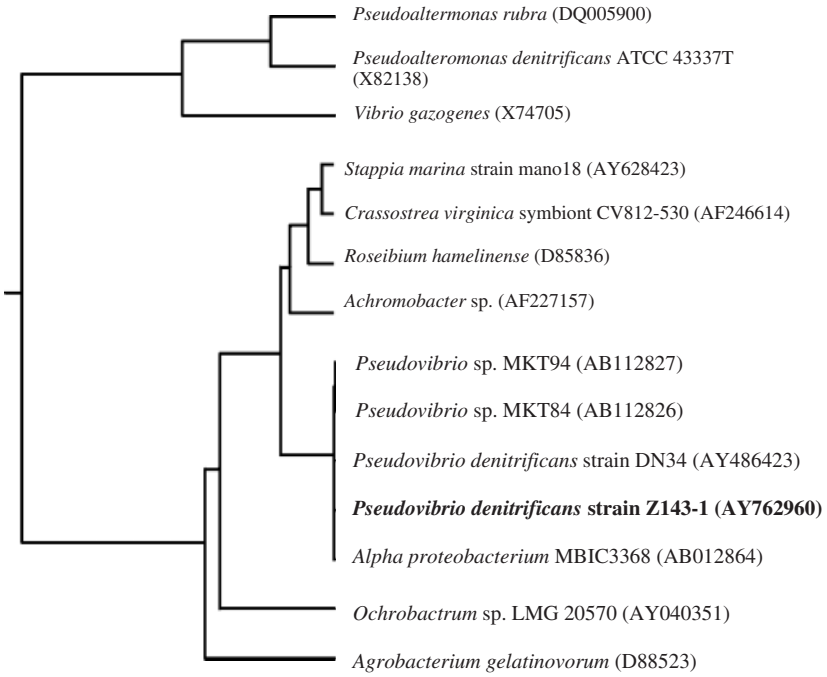

Fig. 1. Phylogenetic tree derived using Fitch–Margoliash and the least-squares method with an evolutionary clock from DNADIST of PHYLIP. Database accession numbers are shown in parentheses.

Table 3. 1D- and 2D-NMR data of heptylprodigiosin ($CDCl_3$, 400 MHz, all δ values in p.p.m. and *J* values in Hz

Position	^{1}H NMR	^{13}C NMR	TOCSY	gHMBC (8 Hz) H to C	gHMBC (3 Hz) H to C	NOESY
1	12.63 br. s		H2, H3, H4			H2, NH-6
2	7.19 m	127.7 d	NH-1, H3, H4			NH-1, H3
3	6.31 m	111.7 d	NH-1, H2, H4			H2, H4
4	6.88 m	117.6 d	NH-1, H2, H3		C3	H3, H8
5		121.7 s				
6	12.63 br. s		H8			NH-1, NH-12
7		148.3				
8	6.04 (d, 1.9)	93.2 d	NH-6	C7, C10		H4, OCH_3-25
9		165.9 s				
10		120.9 s				
11	6.90 br. s	115.9 d		C9, C14	C9, C13, C14	H14, OCH_3-25
12	12.03 br. s		H14			NH-6, H_3-24
13		125.4 s				
14	6.64 (d, 1.9)	129.0 d	NH-12	C13, C16	C13, C15	H11, $H_2$17, $H_2$18
15		128.3 s				
16		146.4 s				
17	2.32 m	25.2 t	$H_2$18, $H_2$19	C15, C16, C18, C19	C14, C16, C18, C19	H14, $H_2$18
18	1.47 (t, 7.42)	29.9 t	$H_2$17, $H_2$19		C20	$H_2$17
19	1.24 m	29.0 t	$H_2$18, $H_2$20	C18, C20	C17, C20	$H_2$17
20	1.18 m	28.9 t	$H_2$19, $H_2$21		C19	
21	1.18 m	31.6 t	$H_2$20, $H_2$22	C22	C19, C23	$H_3$23
22	1.22 m	22.4 t	$H_2$21, $H_3$23	C21, C23	C23	$H_2$23
23	0.82 m	13.9 q	$H_2$22	C21, C22	C21, C22	$H_2$22
24-Me	2.35 s	11.9 q		C15, C16	C15, C16	NH-12
25-OMe	3.95 s	58.5 q		C9	C9	H8, H11

electrophoresis, PCR-based locus-specific restriction fragment length polymorphism or random amplified polymorphic DNA is recommended.

Heptylprodigiosin isolation and identification

Approximately 200 mg of dried hexane extract was obtained from 1 L of culture broth. The ^{1}H-NMR spectrum of the hexane extract indicated a significant fatty acid content. After a series of chromatographic separations as described above, pure heptylprodigiosin was obtained (59% yield, ~118 mg/200 mg dried hexane extract).

The molecular formula of heptylprodigiosin (**1**) was established as $C_{22}H_{29}N_3O$ by HREIMS $[M]^+$ 351.2299 *m/z* (calculated 351.2311). The ^{1}H NMR spectrum ($CDCl_3$, Table 3) exhibited three exchangeable protons [δ 12.63 (2H), 12.03 (1H)], five aromatic H atoms (δ 6.04–7.19), a vinylic proton (δ 6.90, br. s), an aromatic methoxyl (δ 3.95 s), an aromatic methyl (δ 2.35 s), plus an alkyl side chain with seven carbons. The ^{13}C NMR spectrum (Table 3) contained 22 carbons, which were sorted out by a DEPT-135 experiment as two CH_3, one OCH_3, six CH_2, six CH and seven quaternary C atoms. A detailed analysis of HREIMS, NMR and the IR data (ν_{max} 3218, 1673, 1606 and 1247 cm^{-1}) altogether revealed **1** to possess a prodigiosin-type polypyrrole structure. The complete assignment of ^{1}H and ^{13}C signals and the elucidation of the structure of **1** in solution ($CDCl_3$) were established by intensive 2D NMR experiments, COSY, TOCSY, gHSQC, gHMBC and NOESY (Table 3). The TOCSY experiment was particularly helpful in which four spin systems were recognized. The first spin system included the protons of the monosubstituted pyrrole ring (A). It started with the exchangeable NH-1 proton (δ 12.63), which coupled to low-field shifted H-2 atom (δ 7.19 m), which in turn coupled to H-3 (δ 6.31 m). A clear scalar coupling was observed between H-3 and H-4 (δ 6.88 m) and both protons showed a weak coupling with NH-1. The protons of the two trisubstituted pyrrole rings (B and C), namely H-8 (δ 6.04 d) and H-14 (δ 6.64 d), were assigned on the basis of characteristic meta couplings (J = 1.9 Hz) with NH-6 (δ 12.63) and NH-12 (δ 12.03), respectively, completing the second and third spin systems. The final spin system involved the heptyl side chain, i.e., two methylene groups close to the aromatic ring (δ 2.32 H_2-17 and δ 1.47 H_2-18), four highly overlapping methylene protons around δ 1.20 and a typical terminal methyl group (δ 0.82 m). The HMBC correlation experiments acquired at both 8 and 3 Hz and a NOESY experiment also revealed important structural information (Table 3). On the basis of $^2J_{CH}$ coupling, the methoxy function (OCH_3-25, δ 3.95 s) was assigned to C-9 (δ 165.9). An additional NOESY coupling observed between the methoxyl signal and H-8

further supported this assumption. The aromatic methyl group (CH_3-24, δ 2.35 s) had to be attached to C-16 due to its $^2J_{CH}$ and $^3J_{CH}$ couplings with C-16 (δ 146.5) and C-15 (δ 128.3) and an NOE coupling with NH-12. The vinyl proton was assigned to C-11 (δ 6.90) because of its multiplicity (br. s) and the HMBC correlations it showed with C-9, C-13 (δ 125.4) and C-14 (δ 129.0). Finally, the long-range correlations observed from C-17 methylene protons to C-14, C-15, C-16 and an NOE correlation between H-14/H_2-17 firmly established the location of heptyl chain at C-15, thereby proving that **1** is heptylprodigiosin.

The solution properties and proton affinity of prodigiosin analogs are quite complex. Rizzo *et al.* (1999) have previously studied the equilibrium and kinetics of rotamer interconversion of prodigiosin derivatives in solution and reported that, depending on the pH, two isomeric forms (α and β, Fig. 2b) might occur. The equilibrium shifts from the α-conformer at a low pH to a pure β-conformer at a high pH. Additionally, pH modulates the conformational presence of the molecule according to nitrogen protonation on the three pyrrole ring systems. The same authors have also studied the behavior of prodigiosins in different deuterated solvents by NMR (Rizzo *et al.*, 1999). They reported that, in $CDCl_3$, prodigiosin salts only occur as a pure β-conformer, which can easily be recognized by characteristic NOE couplings between the three NH-exchangeable protons (NH-1/NH-6, NH-6/NH-12), H-4/H-8 and H-11/H-14 (Rizzo *et al.*, 1999). In the current study, heptylprodigiosin (**1**) also appeared to be the pure β-rotamer in $CDCl_3$ with protonation on NH-12 (ring C) and showed all spatial correlations expected from the pure β-conformer in the 2D NOESY spectrum. The most indicative NOE couplings are shown in Fig. 2c. This is the first report on full spectral data on heptylprodigiosin in solution.

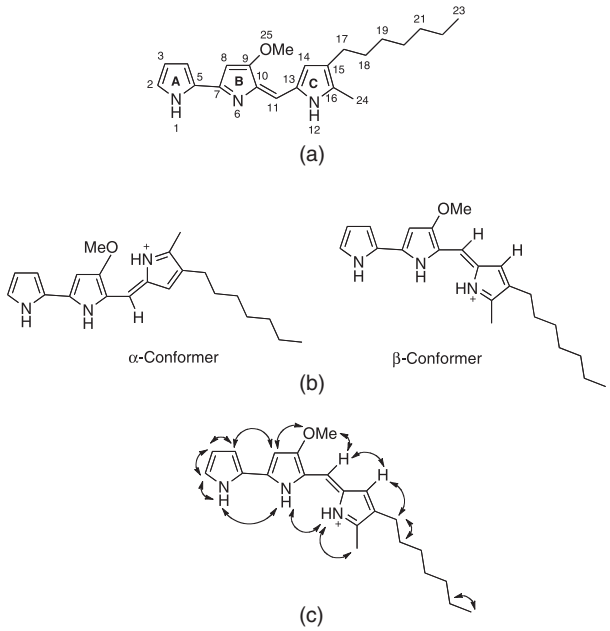

Fig. 2. (a) Structure of heptylprodigiosin, (b) Structures of possible conformers of heptylprodigiosin in solution (adopted from Rizzo *et al.*, 1999 and Melvin *et al.*, 1999) and (c) Key NOESY correlations observed for the β-conformer of heptylprodigiosin in $CDCl_3$.

The structure of the pure compound from Z143-1 in solution ($CDCl_3$) was elucidated (Fig. 2b and c and Table 3) and it was identified as heptylprodigiosin (Fig. 2a). This bright red violet compound was found to be active against MSSA and MRSA (data not shown) in TLC autobiographic overlay assay and, in related studies, it was found to be antimalarial (Lazaro *et al.*, 2002).

Heptylprodigiosin (**1**, 9 mg): Red-purple powder. Infrared (NaCl) ν_{max} 3218 (br), 2920, 2852, 1673, 1606, 1543, 1267, 1995, 1137 and 962 cm^{-1}. LREIMS (70 eV, *m/z*, relative intensity) 351 ($[M]^+$, 100), 282 (13), 252 (10), 192 (10), 185 (33), 175 (8), 93 (32). HREIMS *m/z* 351.2299 (calculated for $C_{22}H_{29}N_3O$ 351.2311) ^{1}H NMR ($CDCl_3$, 400 MHz) see Table 3; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 3. All 2D NMR data are also shown in Table 3.

Conclusion

Pseudovibrio denitrificans strain Z143-1 is by far the only strain of *Pseudovibrio denitrificans* that is reported to produce the bioactive compound heptylprodigiosin. Its potential to produce this compound is quite interesting from a chemical and ecological point of view. From a biological standpoint, its closely related strains were recovered from various sponges and were moreover found to be vertically transmitted via sponge larvae. With its secondary metabolite's structure elucidated, it will be interesting to investigate further the symbiosis of this bacterium with the host organism.

Recent studies (Monge *et al.*, 2007; Soto-Cerato *et al.*, 2007) on prodigiosins prove that it has potential for drug development. Marine microorganisms such as *Pseudovibrio denitrificans* Z143-1 that produce bioactive compounds and can be cultured in the laboratory should be explored for their potential as a renewable source of novel small molecules that could serve as models for new antibiotics and anticancer drugs, for which there is a great need in the world today. Marine compounds isolated from marine invertebrates, such as sponges that are difficult to collect, are often not explored fully because the material collected is inadequate. With sufficient quantity of a pure natural compound from a microbial culture, full structural and conformational studies can be performed, structure analogs can be synthesized, structure–activity relationship studies can be pursued and the specific mechanism of action and molecular target can be elucidated.

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