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The UNDIP-UCSC campaign to culture chemically prolific gram-negative bacteria from Indonesian *Jaspis* sponges

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In honour of Dr. Gordon W. Gribble on the occasion of his retirement from Dartmouth College

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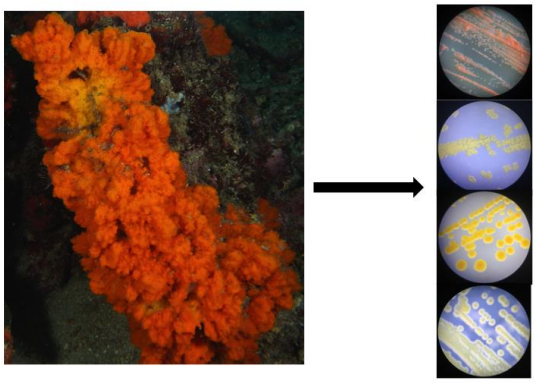
Abstract

Indonesian marine sponges, especially *Jaspis* cf. *coriacea* and *Jaspis splendens* are sources for families of natural products known as the bengamides and jasplakinolides, respectively. Recent research suggests that the biosynthetic origins of these pharmaceutically important compounds may not be the sponges themselves but associated Gram-negative bacteria. Therefore samples of *J.* cf. *coriacea* and *J. splendens* were collected to culture the bacteria responsible for such compounds. Metabolomic analysis was performed on each of the sponges collected to ensure that the bengamides and jasplakinolides were present; therefore ensuring that the biosynthetic machinery required to produce these compounds was intact in the samples. After which Gram-negative bacteria were cultured from the *Jaspis* sponge samples and a total of 43 unique isolates were obtained spanning 21 taxonomic genera and three taxonomic classes.



Keywords: Gram-negative bacteria, Indonesian marine sponges, bioactive marine natural products, bengamides, jasplakinolides

Introduction

The marine environment is the largest habitat on Earth, representing more than 70% of the earth's surface, however it still remains largely unexplored and understudied in comparison to the terrestrial environment.[1] Indonesia is the global epicenter of marine biodiversity, harboring most of the world's marine sponges. The Crews Marine Natural Products Research Group at the University of California Santa Cruz (UCSC) has been engaged in the chemical study of such sponges since the early 1990s and has a repository with hundreds of preserved samples. Recent studies have shown that many natural products found in marine sponges may be biosynthesized by associated microorganisms.[2-5] Furthermore, these sponges provide an unusual environmental niche that hosts diverse microbial taxonomy,[6,7] which in turn may produce unique natural products.

This study is part of a USAID-funded Partnership for Enhanced Engagement in Research (PEER) project entitled, "Enhancing Research Capacity Through a Biotechnology-driven Investigation of Novel Gram-Negative Bacteria from Indonesian Sponges." The project is a collaboration that is co-sponsored by Professor Ocky Karna Radjasa at Diponegoro University (UNDIP) and Professor Phillip Crews at UCSC and focuses on two sponges that have been intensely studied at UCSC for their natural product constituents, *Jaspis* cf. *coriacea* and *Jaspis splendens* (Figure 1). These sponges have repeatedly been a source of the families of bioactive compounds known as the bengamides and the jasplakinolides, respectively (Scheme 1). The rationale for the selection of these sponges is based on our hypothesis that parallel biosynthetic processes are occurring for the natural products from these sponges and from associated Gram-negative bacteria. The bengamides were first discovered by the Crews lab in 1986 and have been shown to have anticancer activity.[8-12] The biosynthetic parallels shown in Figure 2 are stunning, as bengamide E has been isolated from both *J*. cf. *coriacea* (sponge) and *Myxococcus virescens* (Gram-negative bacterium).[2] The jasplakinolides (aka jaspamides) were first isolated by the Crews lab in 1986 and were determined to be potent actin polymerization activators.[13,14] Furthermore, Figure 2 relates the structure of jasplakinolide isolated from *J. splendens* (and other sponges shown) to that of miuraenamide A isolated from *Paraliomyxa miuraensis* (Gram-negative bacterium).[15] Collectively, these parallels in structures suggest that the biosynthetic gene clusters responsible for the bengamides and jasplakinolides are Gram-negative bacterial in origin. Therefore, this study focuses on the culturable Gram-negative bacteria derived from *J.* cf. *coriacea* and *J. splendens* for the purpose of identifying any known or novel natural products they produce under laboratory fermentation conditions.

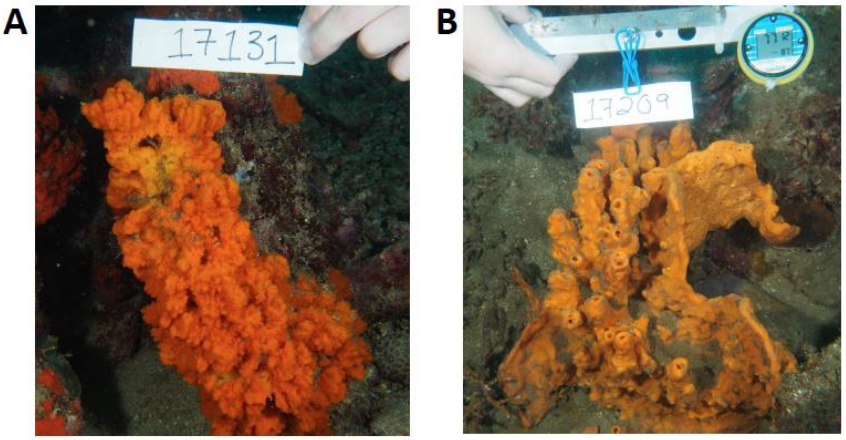
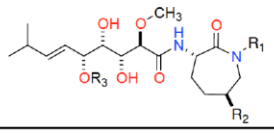
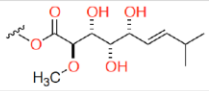
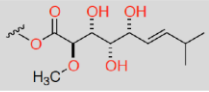
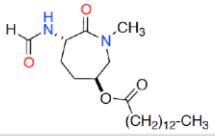
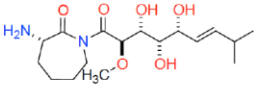
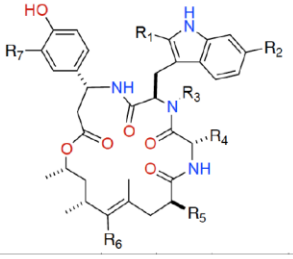
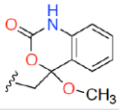
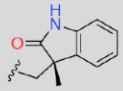
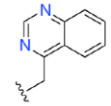
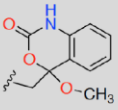


Figure 1. Pre-collection images of (A) *Jaspis* cf. *coriacea* and (B) *Jaspis splendens.*



Bengamide	R_1	R_2	R_3
A	H	$-O_2C(CH_2)_{12}CH_3$	H
B	CH_3	$-O_2C(CH_2)_{12}CH_3$	H
C	H		H
D	CH_3		H
E	H	H	H
F	CH_3	H	H
G	H	$-O_2C(CH_2)_{11}CH_3$	H
H	CH_3	$-O_2C(CH_2)_{11}CH_3$	H
I	H	$-O_2C(CH_2)_{13}CH_3$	H
J	CH_3	$-O_2C(CH_2)_{13}CH_3$	H
K			
L	H	$-O_2C(CH_2)_{11}CH(CH_3)_2$	H
M	CH_3	$-O_2C(CH_2)_{11}CH(CH_3)_2$	H
N	H	$-O_2C(CH_2)_{10}CH(CH_3)_2$	H
O	CH_3	$-O_2C(CH_2)_{10}CH(CH_3)_2$	H
P	H	H	$-CO(CH_2)_{12}CH_3$
Q	CH_3	H	$-CO(CH_2)_{12}CH_3$
R	H	H	$-CO(CH_2)_{14}CH_3$
Y	H	OH	H
Z	CH_3	OH	H
isobengamide E			



Jasplakinolide	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Jasplakinolide	Br	H	CH_3	CH_3	CH_3	H	H
B	Br	H	CH_3	CH_3	CH_3	OH	H
C	Br	H	CH_3	CH_3	CH_3	OH	H
D	Br	H	CH_3	CH_2CH_3	CH_3	H	H
E	Br	H	CH_3	CH_2OH	CH_3	H	H
F	Br	H	CH_3	CH_3	H	H	H
J	Br	H	CH_3	CH_3	H	H	H
M	Br	H	H	CH_3	CH_3	H	H
P		H	CH_3	CH_3	CH_3	H	H
Q	H	H	CH_3	CH_3	CH_3	H	H
R	Br	Br	CH_3	CH_3	CH_3	H	H
S		H	CH_3	CH_3	CH_3	H	H
T		H	CH_3	CH_3	CH_3	H	H
U		H	CH_3	CH_3	CH_3	OH	H
V	Br	H	CH_3	CH_3	CH_3	H	OH

Scheme 1. Structures of bengamide and jasplakinolide analogues.

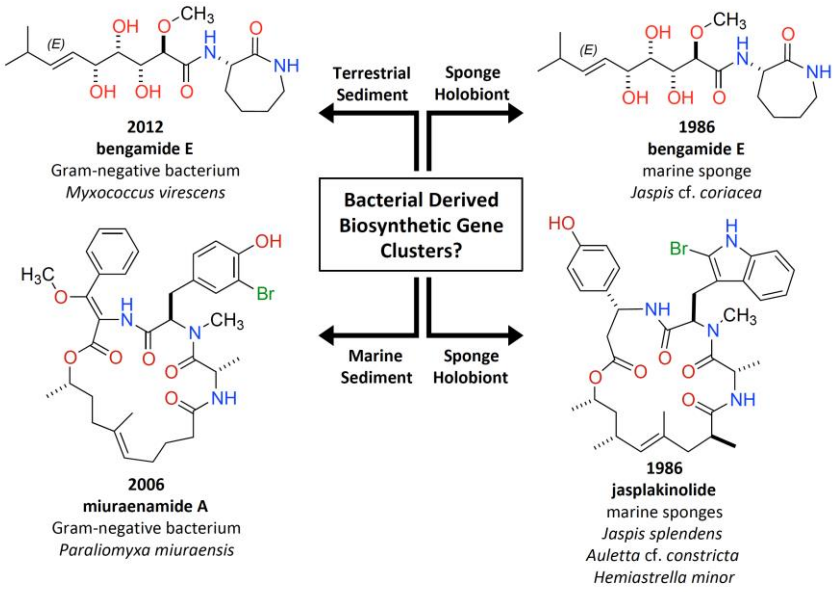


Figure 2. Structures, isolation sources, and discovery years for bengamide E, miuraenamide A, and jasplakinolide.

Result and Discussion

A total of ten samples of *J.* cf. *coriacea* and four samples of *J. splendens* were collected from various sites around Indonesia. Each sample was divided into two fractions; one fraction was frozen and stored at -80 °C to be used for later metabolomic analysis. The other fraction was homogenized and serial dilutions of the homogenate were plated into 48-well plates of either a high or a low nutrient media.

Metabolomic Analysis. Each *Jaspis* sponge sample was extracted and analyzed using ultra high performance liquid chromatography (UHPLC) coupled with high accurate mass spectroscopy (HAMS). The presence of any bengamide or jasplakinolide analogues was putatively determined based on experimental HAMS data for any compounds detected being within ± 5 ppm of their theoretical HAMS data. Ensuring these natural products were present in the sponge samples was essential to ensure that the biosynthetic pathways for these compounds were present and operational. Furthermore, the presence of these compounds in the samples confirmed the taxonomy of the sponges to be *J.* cf. *coriacea* and *J. splendens.* Bengamides D, E, and K were detected in all ten *J.* cf. *coriacea* samples, while A was detected in one sample, F in seven, J and M in three, and Z in four (Table 1). Jasplakinolide, as well as jasplakinolide W and Z was detected in all four *J. splendens* samples, while R was detected in two, and V and C were detected in three.

Table 1. Putative detection of bengamide and jasplakinolide analogues in *Jaspis* sponge samples

Sponge	Taxonomy	Bengamides								Jasplakinolides					
ID		A	D	E	F	J	K	M	Z	Jas.	C	R	V	W	Z
17111	*J.* cf. *coriacea*		x	x			x								
17119	*J.* cf. *coriacea*		x	x			x								
17131	*J.* cf. *coriacea*		x	x	x		x								
17132	*J.* cf. *coriacea*		x	x	x		x		x						
17133	*J.* cf. *coriacea*		x	x	x	x	x	x							
17134	*J.* cf. *coriacea*		x	x	x	x	x	x	x						
17135	*J.* cf. *coriacea*		x	x	x		x		x						
17136	*J.* cf. *coriacea*	x	x	x			x								
17137	*J.* cf. *coriacea*		x	x	x		x		x						
17138	*J.* cf. *coriacea*		x	x	x	x	x	x							
17209	*J. splendens*									x				x	x
17210	*J. splendens*									x	x	x	x	x	x
17211	*J. splendens*									x	x		x	x	x
17213	*J. splendens*									x	x	x	x	x	x

Cultured Gram-Negative Bacteria. All bacteria that grew from the serial dilutions of the sponge homogenates were isolated and pure cultures were obtained by sub-culturing. The potassium hydroxide test was used to distinguish which bacteria were Gram-positive and which were Gram-negative;[16] only the Gram-negative strains were selected for further study. The 16S rRNA gene of these bacteria was sequenced and they were dereplicated using 99% sequence similarity as a cut off for individual strains. From this initial round of culturing, 43 different strains of Gram-negative bacteria were obtained as shown by the bold entries in Figures 3 and 4. The taxonomy for these strains spanned 21 different genera and three difference classes, *Gammaproteobacteria*,

Alphaproteobacteria, and *Flavobacteria*. Natural products are currently being isolated from these Gram-negative strains and will be reported in the future.

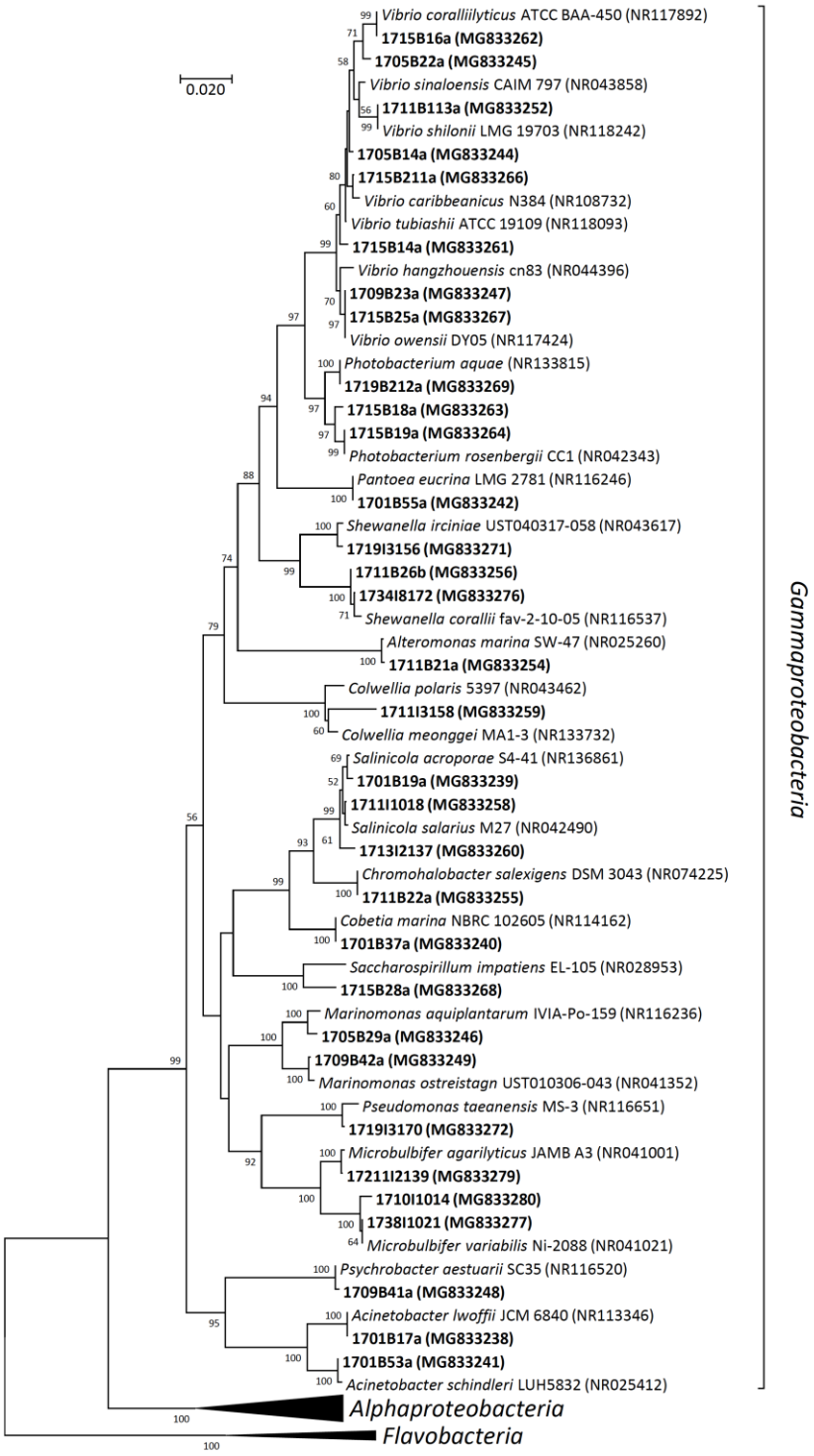


Figure 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences from *Gammaproteobacteria* strains isolated from *Jaspis* cf. *coriacea* and *Jaspis splendens* (in **bold**), as well as reference type strains. Evolutionary distances were computed using the Maximum Composite Likelihood method, a total of 745 nucleotide positions were used. Bootstrap values are expressed as percentages of 1000 replicated; bootstrap values of ≤ 50% are not shown. Bar, 2 substitutions 100 base pairs.

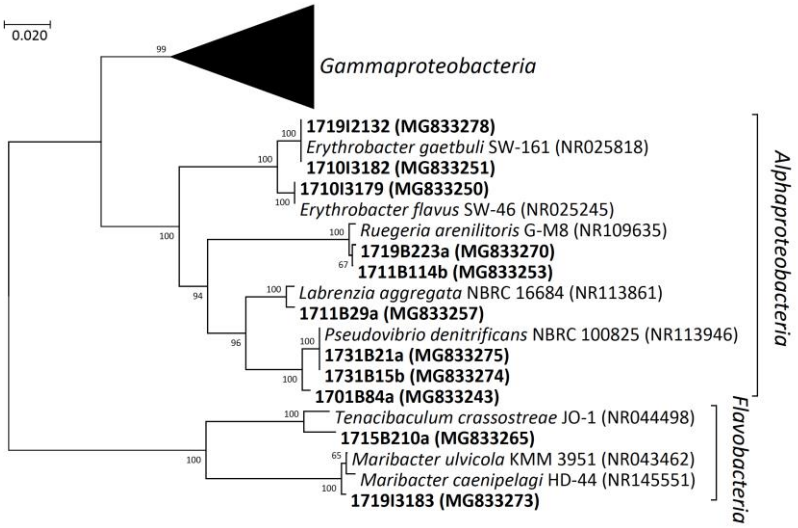


Figure 4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences from *Alphaproteobacteria* and *Flavobacteria* strains isolated from *Jaspis* cf. *coriacea* and *Jaspis splendens* (in **bold**), as well as reference type strains. Evolutionary distances were computed using the Maximum Composite Likelihood method, a total of 745 nucleotide positions were used. Bootstrap values are expressed as percentages of 1000 replicated; bootstrap values of ≤ 50% are not shown. Bar, 2 substitutions 100 base pairs.

Conclusions

This study highlights the first steps taken to identify known and novel natural products from Gram-negative bacteria associated with Indonesian *Jaspis* sponges. From *J.* cf. *coriacea* and *J. splendens* a total of 43 strains of Gram-negative bacteria were obtained that spanned 21 different genera and three difference classes. The task of validating that both sponges re-collected for this study contain natural products known from previous studies has been completed. The subsequent time consuming efforts to identify the natural products produced by these bacterial strains are underway.

Experimental Section

General. All solvent were HPLC grade (Fisher Chemical). UHPLC was achieved using a Phenomenex Luna Omega C18 (150 mm X 2.1 mm, 1.6 µm) column. HAMS was achieved on a Thermo Velos Orbitrap Mass Spectrometer (Thermo Fisher).

Metabolomic Analysis of *Jaspis* Sponges. Approximately 0.5 g of sponge tissue from each *Jaspis* sample collected was ground into a fine powder using liquid nitrogen and a mortar and pestle. Each ground sample was extracted with CH_2Cl_2 (10 mL) dried, then partitioned between 80% aqueous CH_3CN and hexane. The CH_3CN layer was dried, re-suspended in MeOH (1 mg/mL) and analyzed by UHLPC-HAMS a using linear solvent gradient over 20 min from 10:90 CH_3CN:H_2O/0.1% HCO_2H to 95:5 CH_3CN:H_2O/0.1% HCO_2H, followed by 3 min 95:5

CH_3CN:H_2O/0.1% HCO_2H, and an additional 2 min re-equilibration period of 10:90 CH_3CN:H_2O/0.1% HCO_2H at a flow rate of 400 uL/min. The presence of bengamide and jasplakinolide analogues was determined by HAMS.

Culturing Gram-Negative Bacteria from *Jaspis* sponges. A total of ten *J.* cf *coriacea* and four *J. splendens* sponges were collected off the coast of Indonesia in May of 2017 within a depth of 10 - 20 m. Each sample was placed in a 50 mL sterile Falcon® centrifuge tube (Corning®) and rinsed three times with sterile filtered seawater (SFSW) (0.2 um polyethersulfone membrane; Nalgene®) to remove any loosely associated bacteria. A ~ 1 g portion of each sample was frozen and stored in a sterile 15 mL Falcon® centrifuge tube for metabolomic analysis. The remainder of each sample was homogenized in SFSW using an ethanol-sterilized blender. Serial dilutions of the sponge homogenate in SFSW (10^{-2} to 10^{-4}) were prepared and 10 uL aliquots of each dilution series were plated onto either high nutrient Marine Agar (Zobell 2216 HiMedia Laboratories) or low nutrient AVY2 Agar (1 g $CaCl_2 2H_2O$, 2.5 g yeast extract, 1 mg cyanocobalmin, and 15 g agar in 1L of artificial sea water), both media contained 10 µg/mL of cyclohexamide. Plates were incubated at rt and bacteria that grew were purified as single colonies. Gram-negative bacteria were identified using a potassium hydroxide assay[16] and cryopreserved in 20% (v/v) glycerol (VWR) until needed for further processing.

Genomic DNA Extraction and Phylogenetic Analysis. Genomic bacterial DNA was extracted using Extract-N-Amp™ PCR Kit (Sigma-Aldrich) as per manufacturers' instructions. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was achieved using the universal eubacteria 16S rRNA gene primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') and the following thermal cycling parameters: an initial denaturing cycle at 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 54 °C for 1 min, 72 °C for 1.5 min, and a final extension of 72 °C for 10 min. PCR amplicons were assessed by gel electrophoreses in a 1.0% agarose gel containing 0.001% ethidium bromide, at 120V for 30 min (BioRad). PCR products were visualized on a UV transilluminator (BioSpectrum®) and amplicons of the correct size (~1400 bp) were sequenced at the University of California Berkeley Sequencing Facility. Sequences were trimmed and assembled using Vector NTI Contig Express (Invitrogen, Life Tachnologies) and compared to available sequences in the NCBI GenBank database using BLAST.[17] Sequences alignments were prepared using MEGA version 7,[18] phylogenetic histories were inferred using the neighbor-joining method,[19] and evolutionary distances were generated using the Maximum Composite Likelihood method.[20] All positions containing gaps and missing data were eliminated. Bootstrap analysis is based on 1000 resamples datasets.[21] Partial 16S rRNA gene sequences have been deposited in the GenBank database under the accession numbers MG833238 – MG833280.

Acknowledgements

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