

Characterization of a Deep-Sea Sediment Metagenomic Clone that Produces Water-Soluble Melanin in *Escherichia coli*

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Abstract To access to the microbial genetic resources of deep-sea sediment by a culture-independent approach, the sediment DNA was extracted and cloned into fosmid vector (pCC1FOS) generating a library of 39,600 clones with inserts of 24–45 kb. The clone fss6 producing red-brown pigment was isolated and characterized. The pigment was identified as melanin according to its physico-chemical characteristics. Subcloning and sequences analyses of fss6 demonstrated that one open reading frame (ORF2) was responsible for the pigment production. The deduced protein from ORF2 revealed significant amino acid similarity to the 4-hydroxyphenylpyruvate dioxygenase (HPPD) from deep-sea bacteria *Idiomarina loihiensis*. Further study demonstrated that the production of melanin was correlated with homogentistic acid (HGA). The *p*-hydroxyphenylpyruvate produced by the *Escherichia coli* host was converted to HGA through the oxidation reaction of introduced HPPD. The results demonstrate that expression of DNA extracted directly from the environment might generate applicable microbial gene products. The construction and analysis of the metagenomic library from deep-sea sediment contributed to our understanding for the reservoir of unexploited deep-sea microorganisms.

Keywords Deep-sea sediment · Homogentistic acid · 4-Hydroxyphenylpyruvate dioxygenase · *Idiomarina loihiensis* · Melanin · Metagenome

Introduction

The oceans constitute more than 70% of the earth's surface. Work from coastal marine sediments or water is highlighted to portend future direction for deep-sea studies. The deep sea (generally defined by depths >1,000 m) was thought to be a biological 'desert' until submarine hydrothermal systems were discovered along mid-ocean ridges at depths greater than 2,200 m (Nichols et al. 2005). The deep sea is usually characterized by extremely high salinity, darkness, high pressure, and high/low temperature (Deming 1998). Deep-sea environments are centers of unusual biological communities due to the selective pressures on the inhabitants of the ecosystem. Therefore, the deep-sea is considered to be an enormous source of genetic and metabolic microbial biodiversity. Innovative culturing techniques have been employed to gain insight into microbial biochemical process and microbial by-products used for growth and survival in deep sea (Deming 1998). However, the functional analysis and use of most microbial genes were excluded for the majority of the microbial community was not cultivated (Streit et al. 2004). The dilemma of being unable to cultivate the vast majority of the microorganisms present in most microbial niches has forced scientists to develop methods to exploit the genomes of these bacteria for biotechnology without prior cultivation. Given that the metagenomic approach clones the total microbial genome (the metagenome) in culturable bacteria (such as *Escherichia coli*) to discover novel microbial resources, it represents an efficient method of isolating

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novel and useful genes (Lim et al. 2005). Previous studies have shown that the metagenomic approach permits searches for various novel enzymes and metabolites (Lorenz and Eck 2005). However, novel microbial resources from deep-sea sediments have been little explored by metagenomic approach.

Melanins are polyphenolic heteropolymers with a wide range of colors and applications. Due to their chemical composition, melanins have physicochemical properties that allow them to act as ultraviolet absorbers, cation exchangers, drug carriers, amorphous semiconductors, X-ray and γ -ray absorbers, and in some instances, substrates with antioxidant and antiviral activity (Riley 1997; Lagunas-Muñoz et al. 2006). Bacteria are known to produce 3,4-dihydroxyphenylalanine-melanins, the pheomelanins or eumelanins, and melanin-like pigments that are derived from non-nitrogenous phenols (the allomelanins or pyomelanins; Gibson and George 1998). Thus, not all bacterial brown and black pigments are true melanins. Production of melanins by recombinant *E. coli* is useful in industrial processes. Thus, the construction of metagenomic libraries of microorganisms of deep-sea sediments for the identification of novel genes involved in melanin synthesis should be valuable.

In this study, we constructed a metagenomic fosmid library of high-molecular-weight microbial DNA from deep-sea sediment of South China Sea. The fosmid clone fss6 producing melanins was screened, and the genes involved in melanins synthesis were characterized.

Materials and Methods

Sediment Samples

Sediment samples for DNA extraction were collected from the subsurface sediments at water depths of 1,256 m (111° 48.171' E, 17°15.589' N), 1,330 m (110°16.362' E, 17° 09.324' N), 1,575 m (114°07.960' E, 18°45.972' N), and 2,893 m (118°44.856' E, 20°10.074' N) in South China Sea by the Department of Guangzhou Marine Geological Survey. The samples were frozen at -20°C before being processed. To minimize contamination, the superficial parts exposed to the air were discarded when processed.

Extraction and Purification of Environment DNA

DNA extraction was performed using combined physical and chemical treatments described previously (Lai et al. 2007).

The crude DNA fragments of 30–50 kb (size-fractionated by pulsed-field gel electrophoresis, PFGE) were further purified using GELase™ Enzyme Preparation (Epicenter, Madison, WI, USA) according to the manufacturer's

protocol. High-molecular-weight DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol. Then, the pellets were dissolved in Tris–ethylenediaminetetraacetic acid buffer (pH 8.0) for metagenomic library construction.

Metagenomic Library Construction

The size-separated DNAs of 30–50 kb from different locations were pooled equally, and the genomic DNA was end-repaired to blunt end and cloned into the pCC1FOS fosmid vector using the Copycontrol Fosmid Library Production kit (Epicenter), according to the manufacturer's instructions. The ligation mixture was then packed into lambda phages using MaxPlax™ Lambda Packaging Extracts. The packaged DNA was transformed into *E. coli* EPI300™-T1R. The *E. coli* transformants were diluted and plated on Luria–Bertani (LB) agar containing chloramphenicol (12.5 $\mu\text{g}/\text{ml}$). About 250–300 colonies were visible on each plate after incubating overnight at 37°C . The metagenomic library that consisted of 39,600 colonies was constructed by this method and stored at -80°C . Seventy randomly selected fosmid clones were used for determining the average insert size of the library and the polymorphism of the inserts. The fosmid DNA was isolated using alkaline minipreps. Then, the isolated DNA was digested with *Bam*HI (Promega, Madison, USA) and examined by PFGE (Bio-Rad CHEF-DR II) under the conditions of 1% agarose in $1\times$ Tris–acetate- μ (TAE) buffer, 6 V/cm, 5–30 s switch for 16 h.

Subcloning a Pigment-Producing Clone

During the library storage process, we have selected metagenomic clones that altered the phenotype of *E. coli* cloning. The clone, designated fss6, producing red-brown pigment on LB medium was further analyzed by subcloning the responsible genes and identifying the pigment. The fss6 fosmid was extracted using QIAprep Spin Miniprep Kit (Qiagen) and partially digested with *Eco*RI, *Hind*III, *Kpn*I, *Sal*I, and *Sac*I, respectively. Then, the fragments were size-separated by 1% agarose TAE gel electrophoresis. The fragments (1–10 kb) were collected and further purified using Qiaex II Gel Extraction Kit (Qiagen). The purified DNA fragments were ligated into pUC19 (Epicenter) with T4 DNA ligase (New England Biolabs). The ligation products were further transformed into *E. coli* DH5 α . The transformants were grown overnight at 37°C on tyrosine medium (LB containing 0.1% tyrosine) to confirm whether the pigment belongs to melanins. The cloned DNA fragment of one clone (clone fss61) producing red-brown pigment was selected for sequencing using the universal M13-forward and M13-reverse primers. Sequences were analyzed using Editseq, Megalign, and Seqman programs

of the DNASTar Packages. Open reading frames (ORFs) were identified using the ORF Finder program available at the National Center for Biotechnology Information. ORF-conferring pigment generation was further identified by subcloning. Putative complete ORFs (ORF2, ORF3, ORF4, and ORF3 together with ORF4) of *fss61* were amplified using KOD-plus DNA polymerase (Toyobo) with corresponding primers, respectively. These polymerase chain reaction products were purified by the Qiaex II Gel Extraction Kit (Qiagen), digested with *Bam*HI and *Sac*I (Promega), and ligated into pUC19. The ligation products were transformed into *E. coli* cells and screened for pigment production on tyrosine medium.

Pigment Preparation

Purification of melanin produced in liquid cultures of recombinant *E. coli* was performed following a modified procedure from David et al. (1996). One liter of culture broth containing melanin was centrifuged (8,000×g, 15 min, 4°C). The cell pellets were discarded, and the melanin present in the supernatant was precipitated by adjusting the pH to 2.0 with 6N HCl. The precipitated melanin was concentrated by centrifugation (12,000×g, 30 min). Then, the melanin was dialyzed against ddH₂O (8–10 kDa, 24 h) and air-dried. For use in experiment, dried melanin was dissolved in ddH₂O at pH 12. Melanin concentration was determined spectrophotometrically at a wavelength of 400 nm.

Pigment Characterization

Solubility of purified melanin was tested with water, methanol, methanol, acetone, ethanol, chloroform, butanol, ethyl acetate, toluene, and petroleum ether. Reactivity to FeCl₃, KMnO₄, H₂O₂, and NaClO were tested.

The ultraviolet-visible (UV-Vis) absorption spectrum and infrared spectrum were recorded on Shimadzu UV-visible spectrophotometer (UV-2501PC) and Bruker Fourier transformation infrared spectrometer (Equinox 55), respectively.

The free-radical nature of the pigment was investigated by the use of electron paramagnetic resonance (EPR) spectrometer (JES-FE1XG, Jeol, Tokyo, Japan). The parameters for EPR were set as follows: modulation frequency, 100 kHz; modulation amplitude, 10×100; center field, 3,360.0 G; sweep width, 500.0 G; microwave frequency, 9.45 GHz; and microwave power, 4 mW.

Detection of Homogentistic Acid by High-Performance Liquid Chromatography

Exponential phase cultures of recombinant *E. coli* without melanin production in tyrosine medium was used to produce cell-free filtrates. The filtrates (1 ml) were

immediately acidified with 40 µl glacial acetic acid and stored at –20°C until assayed. An aliquot of 5-µl samples was injected onto C18 reverse-phase column (250×4.6 mm) in a HP1100 high-performance liquid chromatography (HPLC; Hewlett-Packard) and UV detector. The pump maintained a flow rate of 0.8 ml/min of the eluting solvent (10% methanol plus 90% 0.01 M KH₂PO₄ solution, pH 3.0). The homogentistic acid (HGA) was detected at 265 nm. Homogentistic acid (Sigma Chemical Co.) was used as standards.

Nucleotide Sequence Accession Number

The nucleotide sequence reported here has been deposited at GenBank under accession number EU196552.

Results

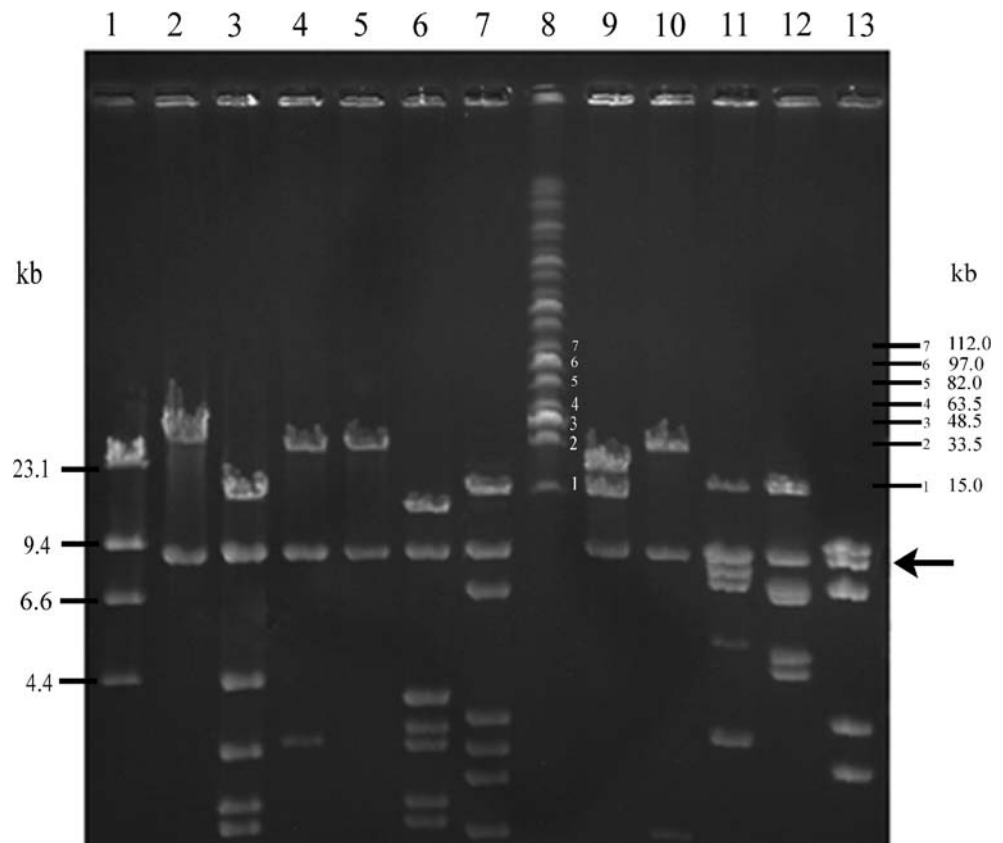
Construction of the Metagenomic Fosmid Library

To obtain highly diverse genes from deep-sea sediments, samples isolated from different sites were pooled with equal quantity and cloned into the fosmid vector. The library that consisted of 39,600 clones was constructed from the deep-sea sediments and maintained for activity-based screening. The average DNA insert size was estimated at 33 kb (ranging from 24 to 45 kb) by analyzing 70 randomly selected clones via PFGE after *Bam*HI digestion (examples are shown in Fig. 1). Restriction analysis of the clones revealed various restriction digestion patterns without any redundant pattern, indicating a high polymorphism of the cloned genes. Given the presence of color is an indication of biosynthetic genes for natural products, the red-brown clone *fss6* was selected for further characterization.

Cloning and Sequence Analysis of Genes for Pigment Production

Since clone *fss6* carried a relatively large insert DNA (approximately 36 kb), the DNA was fragmented by different restriction enzymes and cloned into pUC19 to construct a subsequent library. One clone, designated *fss61*, produced red-brown pigment as clone *fss6* on solid and in liquid tyrosine medium. The inserted DNA of clone *fss61* was further digested with *Sac*I to determine the size of inserted DNA (Fig. 2). The insert DNA of approximately 4 kb was sequenced and analyzed for genetic information. The sequence analysis indicated that *fss61* contains a 4.4-kb insert DNA with 51.8% G + C content. ORF analysis suggested that the *fss61* harbors three complete ORFs (ORF2, ORF3, and ORF4) and one partial ORF (ORF1). The putative proteins encoded by these ORFs

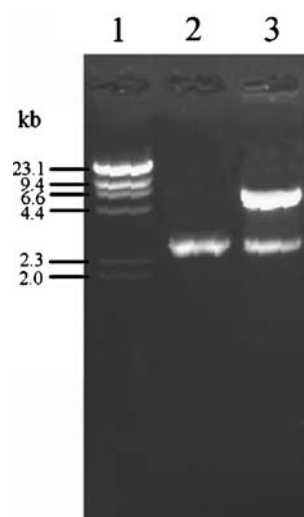
Fig. 1 PFGE of randomly selected fosmid clones digested with *Bam*HI. Lanes 2–7 and 9–13 11 randomly analyzed fosmid clones with sediment DNA inserts, lane 1 lambda DNA/*Hind*III marker, and lane 8 mid-range I PFGE marker



are shown in Fig. 3. Subcloning studies indicated that the recombinant clone carrying ORF2 can produce an identical level of pigment comparing with that of the original clones (fss6 and fss61; Fig. 4). The result suggested that ORF2 is responsible for melanin production.

Sequence analysis of ORF2 (designated *hppd*) indicated that it encodes a protein of 354 amino acids with molecular weight of approximately 40 kDa and isoelectric point of 4.55. A putative purine-rich ribosome-binding site, CGAGG, was observed 5 bp upstream of the start codon.

Fig. 2 Digestion of inserted DNA from clone fss61 with *Sac*I. Lane 1 lambda DNA/*Hind*III marker, lane 2 pUC19 digested with *Sac*I, and lane 3 fss61 with DNA insert responsible for pigment production



The BLASTP search showed that the deduced protein shared high identity with the 4-hydroxyphenylpyruvate dioxygenase from marine bacteria *Idiomarina loihiensis* (identities 81%, accession YP_155112), *Vibrio parahaemolyticus* (identities 75%, accession NP_797728), *Listonella anguillarum* (identities 73%, accession AAL59614), *Aeromonas hydrophila* subsp. (identities 72%, accession YP_857172), *Chromobacterium violaceum* (identities 69%, accession AAQ58643), and *Hahella chejuensis* (identities 67%, accession YP_432268). Multiple alignment of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) with several Gram-negative bacteria and the very distantly related Gram-positive soil bacterium HPPD proteins was presented in Fig. 5. The multiple sequence alignment of HPPD with the homologous HPPDs revealed some of the

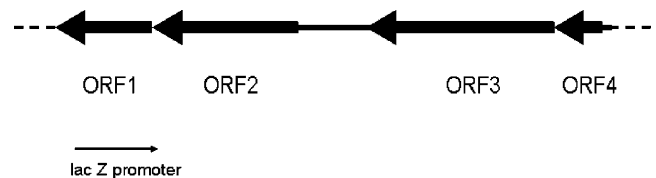


Fig. 3 Schematic diagram of the DNA cloned in fss61. *ORF1* partial similar to a gene encoding homogentisate 1,2-dioxygenase, *ORF2* a gene-encoding putative 4-hydroxyphenylpyruvate dioxygenase characterized in this work, *ORF3* similar to a transcriptional regulator gene, *ORF4* similar to a gene encoding pterin-4-alpha-carbinolamine dehydratase

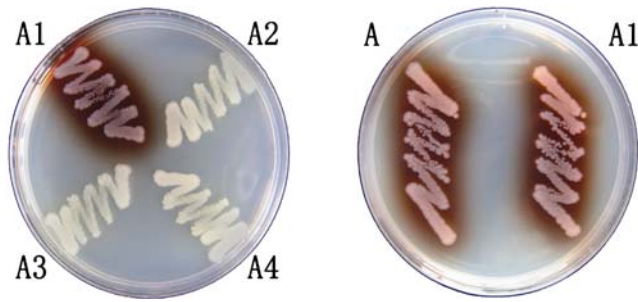


Fig. 4 Analysis of pigment production by *fss61* (A) and recombinant *E. coli* harboring ORF2 (A1), ORF3 (A2), ORF4 (A3), and ORF3 together with ORF4 (A4). All clones grown on tyrosine medium and incubated at 37°C for 24 h

highly conserved residues that are common even in distantly related bacterium. Base on the alignment, most of the conserved residues in the C-terminal domain are essential for catalytic function activity and the presence of four conserved residues, i.e., Pro215, Asn217, Phe334, and Phe338. Further analysis showed that HPPD is rich in tyrosine (17 residues) and histidine (11 residues). Probably, they are potential metal-binding sites of the protein.

Characterization of Melanin Pigment

The pigment produced by clone *fss6* is dissolved in water at pH 10 and precipitated at pH 3. However, it is slightly

HPPD	-----MTDINYNPLQTDGPEFVEYSAPDANGIAALKDLFTKLGPTQVAKHKTKE-----AWLQKQNDINPIVNAE	65
AAL59614	-----MTST-FNPLQTDGPEFVEYTAADPAGIAQLKELFTSLGPAEIAKHSRKE-----AWLYRQGDINPIVNSQ	64
1CJX-C	-----ADLYENPMGLMGPEPIEPASPTPG---TLEPIFEINQPTKVATHRSKN-----VHLYRQGEINLILNNE	61
AAO12525	-----MADIFDNPMGLMGPEPIELASPTPG---VLEPVPQILOPTKVASHRSKD-----VHLYRQGGINLILNNE	62
P69053	-----MGNPNPCLDGPAPLEPSPGDRN---KLGQFPSEMGPAVAHAKNQD-----ITLQKQEIQPIVNAE	60
M59289	-----MASEQNPLOLLQIEPTPATPDLD---FMHVKVPIDPGPKLKKHKQKD-----IVYYKQNDINPLLNE	61
U11864	MTQTTHTPTDARQADPPVKGMDAVVFAVGNAK---QAARYSTAPGMLVAYSQVNGSRETASYVLTNGSARFVLTSTV	77
	: * : : : : : : : : : : : :	
HPPD	TG-----GQAEFPAAKAGHPSVCGMAFRVVDASVAMQHVQANG---AKPFRHTAAGEEPPAVYIGESVLYFVD---	132
AAL59614	PH-----SQAAAFKAVHGSPVCGMAFRVVDASVAMQHVQANG---GREYKTQIGPMELSPAIYIGIGDSLIFVDRYG	134
1CJX-C	PN-----SIASYPAAEHPSPVCGMAFRVKDSQKAYNRALGLG---AQPPIHIDTQPMELNLPATKQIGGAPLYLIDRPG	131
AAO12525	PK-----SIASYPAAEHPSPVCGMAFRVRNAEAYARALEGLG---AQPVEIETQPMELRRLPAIKQIGGAPLYLIDRPG	132
P69053	SH-----CQAEAHASTHGPGACANGFKVDAAAFQHASARG---GIAFDQAPBAN-HGLPAIQAIIGSVLYFVD---	126
M59289	KQ-----GFSAQFARTGPAISMGWRVEDANFAFEGAVARG---AKP--AADEVKDLPPAIYQIGDSLIFPIDTQ	129
U11864	IKPATPWGHPFLADHVAEHGQGVVDLAIEVPDARAARAYAIEHGARSVAEPPYELKDEHGTVVLAAIATYKTRHTLVDRTG	157
	.. ** . : . * : : . . . : . . . : . . . : . . . : . . . : . . . : . . . :	
HPPD	--KDIYTNDPDPFYDNWEAJOKKDHAAAGLYEVDLTLTENVFRGNMIDWAGPYERIGNPREIRYPD---IEGKLTGLLSRAMTA	207
AAL59614	--SQSIYDVDFKPYADANQRMTDANVGLPEIDLTLTENVYRQGMIDWSGPYERIGNPREIRYPD---IEGKLTGLVSRAMTA	210
1CJX-C	EGSSIIDIDFPVYLEGVER--NPVGAQLKVIDLTLTENVYRGMVYMANPYEKLFPNPREARYPD---IKGEYTGTSKAMSA	206
AAO12525	EGSSIIDIDFPNPIEGVDR--NPVGAQLKIIDLTLTENVYRGMAYWAGPYEKLFPNPREIRYPD---IKGEYTGTSKAMTA	207
P69053	---EEHQPPSHETWNTISPEPVVGNGLTALIDLTLTENVYRGMIDWAGPYEKLFPNPREIRYPD---IKGKMTGLVSRALGS	199
M59289	DDNNIYTSDF--EALDEPIITQEKGFIEVDLTLTNNVHKGTMEYWSNFPYKIDIFGTFEVRYPD---IKGSQATALISYALRS	203
U11864	-YDGPYLPQYVAAAPIVEP--PAHRTQOAIIDLCVGNVELGRMNEWVGFYKVMGPTNKKEPVGDDIATEYSALMSKVVAD	234
	: : : ** . * * . . . : . . : : . . . : . . . : . . . :	
HPPD	PCGKIRIPANE--SHDDK--SQIEEYLREYNQEGIGIQIALTSDDIYKTVRDRAIGNDPMTTP--DTYYAKIDERVEGHEENV	284
AAL59614	PCGKIRIPANE--SSDDK--SQIEEPIREYNQEGIGIQIALTSDDIYQTVRTLRERGNDPMTTP--DTYYEKVSRVGEHQEDV	287
1CJX-C	PDGMIRIPANE--ESSKGA--QOIEEPLMQFNQEGIQIVAPLTDLLKTDALKKIGNRPMTAPPDQTYEENLEERLPQHGEPV	285
AAO12525	PDGMIRIPANE--ESSKGA--QOIEEPLMQFNQEGIQIVAPLTDLLKTDALKKGYGNRPMTAPPDQTYEENLEERLPQHGEPV	286
P69053	PCGKIRIPANE--SKDDL--SQIEEPLHKEYHGEIGIQIALTNNDIYKTVNGLRKGQVKPLDVP--DTYYEMINDRLPMHKEPL	276
M59289	PDGSPCIPANE--CKGDDR--NQIDEYLKEYDQPGVQDLAPRSRDIVASLDAMEGSSIQTLDI--PEYYDTIFEKLPQVTEDR	281
U11864	GTLLKVKFENEPALAKKKSQIDEYLEPFGGAGVQIALNLTGDIVETVRTMRAAGVQPLDTP--DSYYDTLGENVGDTRVPV	313
	: : : ** : : * :	
HPPD	DELRELQILIDGAPMKDG--ILLQIPTQTVIG--PVFPHI IORKNGEFGPEGNFKALFESIEEDQIRRGVLSDA-	354
AAL59614	DQLKALRILIDGAPMKDG--ILLQIPTQTVIG--PVFPHI IORKNGEFGPEGNFKALFESIEEDQIRRGVLSDA-	357
1CJX-C	DQLQARGILLDGSSEVQDGRLLQIPSETLNG--PVFPHI IORKGDDGFGPEGNFKALFESIERDQVRRGVLATD-	357
AAO12525	DQLQARGILLDGASQPEDKRLLQIPSETLNG--PVFPHI IORKGDDGFGPEGNFKALFESIERDQVRRGVLATD-	358
P69053	NQLBAEKILIDGADPKD--GLLQIPTENIFG--PVFPHI IORKNGEFGPEGNFKALFESIERDQVRRGRLKELS	348
M59289	DRIKHHQILVDGD--EDG--YLLQIPTKQLPG--PIPIPI IORKNNGFGPEGNFKALFESIERDQVRRGVL----	346
U11864	DTLRELKILADRD--EDG--YLLQIPTKPVQDRPTVPFPHI IERHGSNGFGKGNFKALFESIERDQEKRGNL----	380
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Fig. 5 Amino acid sequence blocks conserved in the deduced amino acid sequences of HPPD and its homologs. The amino acids of HPPD are aligned with the sequences of the HPPD proteins of *Listonella anguillarum* (accession AAL59614), *Pseudomonas Fluorescens* (accession 1CJX-C), *Pseudomonas putida* (accession AAO12525), *Legionella pneumophila* (accession P69053), *Shewanella colwelliana*

(accession M59289), and *Streptomyces avermitilis* (accession U11864). The amino acid residues suggested to be active iron atom (His162, His240, and Glu319) and play an important role in substrate binding, positioning, and directing catalysis (Pro215, Asn217, Phe334, and Phe338) are boxed. The identical, strong similarity and weak similarity are marked by an asterisk, colon, and dot, respectively

soluble in either methanol or methanal, insoluble in acetone, ethanol, chloroform, butanol, ethyl acetate, toluene, and petroleum ether. It was retained in the dialysis tubing (8,000 molecular weight exclusion). The pigment was profoundly bleached by the action of H_2O_2 and NaClO . A brown flocculent precipitate was formed after FeCl_3 was added. In the presence of KMnO_4 , the color of the pigment solution turned green from brown. The chemical characteristics of the extracted pigment showed similarities to those of melanin.

The absorption spectrum of the pigment revealed a featureless decreasing profile over the range 190–900 nm just like melanin. The Fourier transform infrared spectrum of the pigment showed the peaks at the following wave length: A peak at 3,400–3,300 cm^{-1} is ascribed to OH or NH group stretching; peak at 2,960–2,860 cm^{-1} ascribed to CH_2 , CH_3 , and NH group stretching; peak at 1,650–1,600 cm^{-1} ascribed to aromatic C=C conjugated with C=O and/or COO^- group stretching; peak at 1,310–1,450 cm^{-1} ascribed to stretching of amino groups and CH_2 groups of aliphatic radicals, and may be include phenolic C–O–H bending. Based on the EPR spectra of the pigment (data not shown), it is possible to state the stable free radical nature of paramagnetic centers responsible for the EPR signal of the pigment. Thus, the physico-chemical characteristics of the pigment are those typically described for melanins.

HPLC Analysis for HGA

HPLC analysis for HGA from the cell-free filtrate of cultures showed that HGA was produced by recombinant *E. coli* strains before melanin was produced (Fig. 6). Higher peak without shoulders was produced at the retention time point of standard HGA when standard HGA solution was added into the cell-free filtrate. *E. coli* harboring vector pUC19 (control) did not produce pigment and HGA. Therefore, HGA is the product of ORF2 and should be the precursor of melanin.

Discussion

In this study, a metagenomic library that consisted of 39,600 clones was constructed from deep-sea sediment samples (depths ranging from 1,256 to 2,983 m). The metagenomic library represented about 1,300 Mbp of genomic DNA in deep-sea sediments. Therefore, the construction and analysis of the metagenomic library from deep-sea sediment contributed to our understanding for the reservoir of unexploited deep-sea microorganisms.

The clone *fss6* producing red-brown pigment was isolated and further characterized in the study. Based on

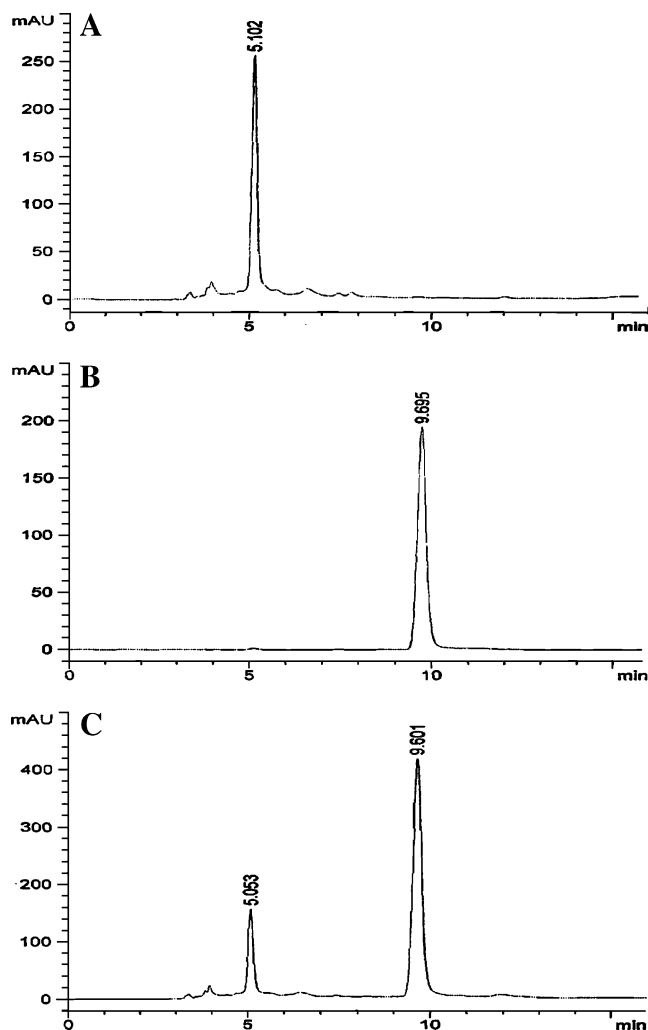


Fig. 6 Detection of homogentistic acid (HGA) by HPLC. **A** Supernatant of a culture of *E. coli* harboring pUC19 (control), **B** standard solution of HGA, and **C** supernatant of a culture of *E. coli* harboring ORF2

the physico-chemical characteristics of the pigment, the red-brown pigment was identified as melanin. The cloned gene (ORF2) encoding putative 4-hydroxyphenylpyruvate dioxygenase (HPPD) was responsible for the production of melanin in *E. coli*. The HPPD catalyzes the conversion of 4-hydroxyphenylpyruvate to HGA. This reaction is the second step of a metabolic pathway that generates acetoacetate and fumarate from L-tyrosine (Knox and Edwards 1955). Given that ketogenic and glucogenic products have a direct energetic contribution, the transformation catalyzed by HPPD showed both agricultural and therapeutic significance (Keon and Hargreaves 1998). Although the HPPD genes have been cloned from the metagenomic library of soil microbial DNA, the gene isolated from deep-sea sediment showed no similarity to the reported soil HPPD genes (MacNeil et al. 2001; Gillespie et al. 2002). The deduced amino acid sequence of the HPPD

shares high identity with that of deep-sea bacterium *I. loihiensis*. Probably, the insert DNA of clone *fss6* originated from a member of this genus. The members of *Idiomarina* could tolerate different extreme marine environments and were isolated from deep-sea hydrothermal vents, deep-sea water, and hypersaline water of solar saltern; however, they do not produce melanin (Ivanova et al. 2000; Brettar et al. 2003; Donachie et al. 2003; Martínez-Cánovas et al. 2004; Choi and Cho 2005). The *I. loihiensis* genome revealed that the bacteria derive carbon and energy primarily from amino acid metabolism rather than sugar fermentation (Hou et al. 2004). The HPPD enzyme is likely to be involved in tyrosine metabolism.

The known pathway for the catabolism of tyrosine to HGA in mammals and bacteria is that tyrosine is converted to *p*-hydroxyphenylpyruvate (*p*-HPP) through a transamination reaction. Then, *p*-HPP is converted to HGA through the oxidation of HPPD (Lindstedt and Odelhög 1987; Lindstedt et al. 1977). In the study, the transaminase for the initial step should exist in clone *fss6*. Three aminotransferase capable of converting tyrosine into *p*-HPP have been identified in *E. coli* (Pittard 1987). We propose that tyrosine is first catabolized by aminotransferases of *E. coli* host, then, the produced *p*-HPP is converted to HGA by the introduced HPPD gene products. Lastly, the accumulation and polymerization of HGA results in the formation of melanin. The results demonstrate that the chimeric metabolic pathway could generate different chemical structures, and expression of DNA extracted directly from the environment might find previously uncharacterized natural products.

The general limitations of metagenomic cloning and heterogenous expression in *E. coli* have been found (Daniel 2005). Developing the technology such as expression in hosts with different expression properties should greatly enhance the ability to detect novel biocatalysts and natural products from unusual and extreme environments. The metagenomic approach should increase the likelihood of future discoveries of novel natural products.

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