

RESEARCH LETTER – Environmental Microbiology

Aerobic microbial communities in the sediments of a marine oxygen minimum zone

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One sentence summary: Aerobic microorganisms in marine oxygen minimum zone sediment.

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Editor: Rich Boden

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ABSTRACT

The ecology of aerobic microorganisms is never explored in marine oxygen minimum zone (OMZ) sediments. Here we reveal aerobic bacterial communities along ~3 m sediment-horizons of the eastern Arabian Sea OMZ. Sulfide-containing sediment-cores retrieved from 530 mbsl (meters beneath the sea-level) and 580 mbsl were explored at 15–30 cm intervals, using metagenomics, pure-culture-isolation, genomics and metatranscriptomics. Genes for aerobic respiration, and oxidation of methane/ammonia/alcohols/thiosulfate/sulfite/organosulfur-compounds, were detected in the metagenomes from all 25 sediment-samples explored. Most probable numbers for aerobic chemolithoautotrophs and chemoorganoheterotrophs at individual sample-sites were up to 1.1×10^7 (g sediment)⁻¹. The sediment-sample collected from 275 cmbsf (centimeters beneath the seafloor) of the 530-mbsl-core yielded many such obligately aerobic isolates belonging to *Cereibacter*, *Guypparkeria*, *Halomonas*, *Methylophaga*, *Pseudomonas* and *Sulfitobacter* which died upon anaerobic incubation, despite being provided with all possible electron acceptors and fermentative substrates. High percentages of

Received: 15 May 2020; Accepted: 23 September 2020

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metatranscriptomic reads from the 275 cmbsf sediment-sample, and metagenomic reads from all 25 sediment-samples, matched the isolates' genomic sequences including those for aerobic metabolisms, genetic/environmental information processing and cell division, thereby illustrating the bacteria's *in-situ* activity, and ubiquity across the sediment-horizons, respectively. The findings hold critical implications for organic carbon sequestration/remineralization, and inorganic compounds oxidation, within the sediment realm of global marine OMZs.

Keywords: aerobic microorganisms; sulfur-oxidizing chemolithotrophs; marine hypoxic zone sediments; Arabian Sea oxygen minimum zone; metaomics; genomics

INTRODUCTION

Microbial ecology of marine oxygen minimum zones (OMZs; dissolved O₂ perennially < 20 μM) has profound influence on oceanic biodiversity, productivity and fixed-nitrogen loss (Wright, Konwar and Hallam 2012). However, for OMZs distributed across the global ocean, microbiome architecture of the sediments are less explored than those of the water-columns (Ulloa et al. 2012; Bertagnolli and Stewart 2018). Most studies on the sediments, in turn, are centered on geochemical manifestations of microbial processes while few involve direct characterization of microbiomes (van der Weijden, Reichart and Visser 1999; Schulte, Mangelsdorf and Rullkötter 2000; Cowie et al. 2014; Yoshinaga et al. 2015; Maltby et al. 2016; Cavan et al. 2017; Orsi et al. 2017; Fernandes et al. 2018). Albeit hypoxic, OMZ waters afford sufficient dissolved O₂ for the sustenance of aerobic metabolisms (Lam et al. 2009; Canfield et al. 2010; Garcia-Robledo et al. 2017). OMZ sediments, on the other hand, have shallow O₂-penetration depth (Breuer et al. 2009) due to high flux of labile organic matter across the sea-bed leading to rapid consumption of O₂ (Cavan et al. 2017; Jessen et al. 2017). In this scenario, the ecology of aerobic microorganisms is never investigated in OMZ sediments, even though potential *in-situ* aerobic metabolisms can have significant impact on the remineralization/sequestration of buried organic matter within this vast marine-sediment system.

Among all perennial OMZs of the marine realm, the one in the Arabian Sea (ASOMZ) is the thickest (vertical expanse: ~1.2 km) and largest (total area: ~3.2 × 10⁶ km²) (Moffitt et al. 2015; Acharya and Panigrahi 2016). Here we explore the microbiome for potential aerobic communities in two sulfide-containing sediment-cores (each ~300 cm long) collected from about the midpoint of the upright span of the eastern segment of ASOMZ (water-depths: 530 and 580 mbsl), where anaerobic processes of the carbon-sulfur cycle are already known to be highly active (Fernandes et al. 2018). Presence and activity of aerobic microorganisms including sulfur-oxidizing chemolithotrophs were revealed using metagenomics, culture-based approaches, genomics and metatranscriptomics. The findings hold critical implications for carbon-sulfur cycling in sediments underlying all hypoxic marine water masses.

MATERIALS AND METHODS

Study sites and sampling

The sediment-cores SSK42/5 and SSK42/6, which constitute the raw material of the current investigation (Fig. 1), were retrieved aboard RV Sindhu Sankalp (research cruise SSK42) from ASOMZ sites located within the western Indian continental shelf territory, at GPS coordinates 16°49.88' N, 71°58.55' E (580 mbsl water-depth) and 16°50.03' N, 71°59.50' E (530 mbsl water-depth), respectively. The two sediment-cores were sampled at 15–30 cm resolution, as described previously (Fernandes et al. 2018; Mandal et al. 2020a). To avoid exposure of the native microflora to aerial O₂ and/or oxidation of the reduced chemical substances

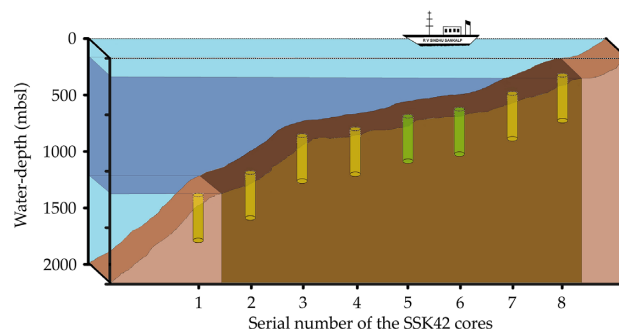


Figure 1. Schematic diagram showing the locations of the currently-explored sediment-cores SSK42/5 and SSK42/6 in the context of the ASOMZ and the other SSK42 cores studied previously by Fernandes et al. (2018). Water-depth is plotted in scale along the vertical axis of the diagram, while distances between the cores represented along the horizontal axis are not in scale. The OMZ is indicated by blue-gray shade; both the upper and lower oxyclines are indicated by light turquoise shade; sediment-horizons underlying the different water-zones are indicated by different shades of brown.

present in the sediments, only a C-shaped portion (30 cm in length) of the core-liner (made of poly vinyl chloride) was removed in one go. Throughout the sampling process, this 30 cm exposed core-length was thoroughly bathed with highly pure N₂ supplied from a number of nitrogen generators and corresponding plungers. Furthermore, to remove all probable contaminations from the inner surface of the core-liner and/or the seawater that had filled the empty core during its lowering through the water-column, superficial one centimeter of the sediment under sampling was first shaved off aseptically, along the circumference of the core.

For each sediment-depth explored in a core, three sample-replicates, designated for duplicate metagenome analyses and one metatranscriptome analysis, were collected in separate screw-capped bottles; in addition, two more replicates were taken for culture-based studies. All sample-containing bottles were bathed with high-pure N₂, sealed by Parafilm (Bemis Company Inc., Neenah, USA) and stored in the cold: sample-replicates earmarked for culture-independent studies were stored at -20°C while those for culture-based investigations were kept at 4°C. Over the study, sample-bottles were consistently stored at their assigned temperatures and opened only inside an H35 Hypoxystation (Don Whitley Scientific, Bingley, UK) having 0% O₂ partial pressure and 75% humidity; after every use, the bottles were screw-capped and then sealed afresh with new Parafilm.

Metagenome/metatranscriptome/genome sequencing

Onboard SSK42, metagenomes were extracted from sediment-samples with the help of PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA), while metatranscriptome was extracted by RNA PowerSoil Total RNA Isolation Kit (Mo Bio Laboratories Inc.) from sample-replicate treated with RNAlater (Ambion Inc., Austin, USA). Genomes of bacterial strains were

isolated with the help of HiPurA Bacterial Genomic DNA Purification Kit (Himedia, Mumbai, India).

The Ion Proton platform (Thermo Fisher Scientific, Waltham, USA) was used to individually sequence the duplicate metagenomes obtained for each of the 25 sample-sites explored. Bacterial genomes were sequenced using the Ion S5 platform (Thermo Fisher Scientific). Paired-end (2×150 nucleotide) sequencing of metatranscriptome was carried out on a HiSeq4000 platform (Illumina Inc., San Diego, USA). Ribo-Zero Gold (Illumina Inc.) was used to remove rRNAs from the metatranscriptome before its sequencing. To remove any rRNA-related read which could still be present in the sequence dataset obtained, each of the total 26 579 343 read-pairs generated were searched against the SILVA database of rRNA gene sequences (Quast et al. 2013), using Bowtie2 v.2.3.4.3 (Langmead and Salzberg 2012) in default mode. In the process, 26 496 769 read-pairs remained in the rRNA-sequence-free dataset, which was eventually used for all metatranscriptomic analyses.

Metagenome/metatranscriptome/genome analysis

All high-quality reads (Phred score cut-off 20) present in the metagenomic dataset-pair of each sediment-community were co-assembled into contigs, and then searched for genes (open reading frames), using Megahit v1.2.x (Li et al. 2015), MetaQUAST (Mikheenko, Saveliev and Gurevich 2016) and MetaGeneMark (Zhu, Lomsadze and Borodovsky 2010), as described by Roy et al. (2019). The python script rnaspades.py, available within SPAdes 3.13.0 (Nurk et al. 2013), was utilized to assemble the metatranscriptomic sequence dataset devoid of rRNA reads; Prodigal v2.6.3 (Hyatt et al. 2010) was then used to predict genes in > 100 bp contigs. Genomes of bacterial strains were assembled and annotated using the softwares SPAdes 3.13.0, QUAST (Gurevich et al. 2013), Bandage (Wick et al. 2015) and PGAP (NCBI, USA), as described by Sen et al. (2020). The gene-catalogs obtained from the assemblies of the metagenomes, metatranscriptome, or genomes were functionally annotated by searching against the database EggNOG v5.0, using the software EggNOG-mapper and the algorithm HMMER (Huerta-Cepas et al. 2016). Total metagenomic, or rRNA-sequence-free metatranscriptomic, reads available for the different sediment-samples were mapped onto the individual bacterial genomes, or specific gene-catalogs curated manually from the bacterial genomes, using Bowtie2 v.2.3.4.3 in default mode.

Most probable number (MPN) of aerobic bacteria

Most probable number of live cells of aerobic, chemoorganoheterotrophs and sulfur-chemolithoautotrophs in the different sediment-samples was calculated, as described previously (Sutton 2010), using 10-fold dilution series (and three tubes per dilution) of aerobic slurry-cultures (Figure S1, Supporting Information) in Luria broth (pH 7.0), and artificial sea water (ASW) supplemented with 40 mM S thiosulfate (ASWT; pH 7.5), respectively.

Isolation of aerobic bacteria and their characterization

Microbial consortia were aerobically enriched from the sample originating at 275 cmbsf sediment-depth of the core SSK42/6. The following broth media were used for that purpose: (i) ASWT, (ii) MSTY (pH 7.0) that contained mineral salts (MS) plus thiosulfate (20 mM) and yeast extract (500 mg/L), and (iii) ASWTY (pH 7.5), which was an ASWT-variant supplemented with yeast

extract (500 mg/L). Sediment-sample (5% w/v) was added to the three different culture media contained in separate cotton-plugged conical flasks having 60% headspaces filled with air, and incubated aerobically at 15°C. After the phenol red indicator of the broth media changed its color to yellow on account of sulfuric acid formation (from thiosulfate), isolation of aerobic bacterial strains was carried out via serial dilution and spreading onto corresponding agar plates, aerobic incubation at 15°C, and finally iterative dilution streaking. Strains were maintained in their respective medium of isolation; only the *Methylophaga* strains, despite being isolated in ASWT, had to be maintained in ASWM [i.e. ASW plus 0.3% (v/v) methanol] since their growth weakened after six consecutive ASWT sub-cultures. Sulfate, tetrathionate and thiosulfate concentrations in the culture media were estimated by gravimetric precipitation, cyanolysis and iodometric titration respectively (Kelly and Wood 1994; Alam et al. 2013); the assay results were validated by ion chromatography (Rameez et al. 2020). New isolates were classified up to the lowest taxonomic category identifiable, using methods described by Saha et al. (2019).

Tests for anaerobic/fermentative growth/survival

New isolates were incubated within the H35 Hypoxystation (having 75% humidity and 0% partial pressure of O_2) to test anaerobic growth/survival in their respective maintenance-media (Table 1) supplemented with $NaNO_3$ (4 mM; Straub and Buchholz-Cleven 1998), Fe_2O_3 (125 mM; Lovley and Phillips 1986), MnO_2 (1 mM; Myers and Nealon 1988), Na_2SO_4 (10 mM; So and Young 1999), $(CH_3)_2SO$ (56 mM; Oren and Trüper 1990) and $(CH_3)_3NO$ (27 mM; Oren and Trüper 1990) as electron-acceptors, provided both as mixture of all six compounds and as single respiratory substrate. Isolates were also tested for anaerobic growth in their respective maintenance media supplemented with humic acids (17 mg/mL; Benz, Schink and Brune 1998), or humic acids (17 mg/mL) and Fe_2O_3 (40 mM) in combination (Benz, Schink and Brune 1998). Isolates which died in these culture conditions were tested for fermentative growth/survival. Isolates that could not grow aerobically/anaerobically in the fermentative medium generally used for lactobacilli (Mercier et al. 1992) were subsequently provided with sodium pyruvate (5 g/L) as the fermentative substrate. Since fructose is the only multi-carbon compound utilized by known *Methylophaga* species (Janvier et al. 1985), the current isolate belonging to this genus was tested for fermentative growth in ASWM supplemented with 0.3% (w/v) fructose.

Details of nucleotide sequencing, MPN determination, media composition/preparation, culture inoculation, growth monitoring and sulfur-chemolithotrophy test are given in Supplementary Methods.

RESULTS

Genes and mRNA transcripts related to aerobic metabolisms are abundant in ASOMZ sediment communities

Metagenome analysis for the microbial communities occurring along both SSK42/5 and SSK42/6 revealed footprints of various aerobic metabolisms. When the metagenomic sequence dataset-pair of each sediment-sample (Tables S1 and S2, Supporting Information) was co-assembled and annotated the 25 contig-collections obtained were found to encompass genes concerned with aerobic respiration by low- as well as high- O_2 -affinity cytochrome oxidases. These included genes for aa_3 -type

Table 1. The nine bacterial species isolated from 275 cmbfs of SSK42/6, and the anaerobic/fermentative growth properties of their representative strains.

Identification up to lowest taxonomic level possible	Bacteria isolated in ASWT			Bacteria isolated in ASWTY			Bacteria isolated in MSTY		
	<i>Guyprankeria</i> sp.	<i>Methylophaga</i> sp.*	<i>Sulfobacter</i> sp.	<i>Halomonas</i> sp.	<i>Stenotrophomonas</i> sp.	<i>Halomonas</i> sp.	<i>Pustillimonas ginsengisoli</i>	<i>Pseudomonas butzandensis</i>	<i>Cereibacter changlaensis</i>
Total number of strains isolated	6	2	8	2	1	3	2	2	1
Name of the representative strain	SB14A	SB9B = MTCC12599	15WGC = MCC3606	15WGF = MCC3301	SBPC3	SBBP1	SB03 = MTCC12559	SBBB = MTCC12600	SBBC = MTCC12557
16S rRNA gene/whole genome sequence accession no. of the representative strain	LN999387/SWAW01000000	LN999390/SSK.S01000000	LT607023/SZNL01000000	LT607031/SSXT01000000	LN999400	LN999401	LN999404	LN999396/SWAV01000000	LN999397/SW.AU01000000
Media used to check anaerobic growth [‡]	ASWT	ASWM	ASWTY	ASWTY	ASWTY	ASWTY	MSTY	MSTY	MSTY
CFU# present per mL culture at 0 h	6.1*10 ⁴	6.1*10 ⁴	2*10 ⁴	3.8*10 ⁴	7.8*10 ⁴	4.5*10 ⁴	1.9*10 ⁴	5.1*10 ⁴	2.6*10 ⁴
CFU# present per mL culture after 10 days incubation	2.8*10 ³	0	0	0	1.6*10 ⁷	9*10 ⁷	2.3*10 ⁷	0	0
Media used to check fermentative growth	ASWT	ASWMF	ASWTYP	ASWTYP	NA	NA	NA	MSTYP	MSTYP
CFU# present per mL culture at 0 h	7.9*10 ⁴	7.8*10 ⁴	2.2*10 ⁴	3.5*10 ⁴	NA	NA	NA	6*10 ⁴	2.5*10 ⁴
CFU# present per mL culture after 5 days incubation	NR	2.8*10 ⁴	0	0	NA	NA	NA	1.6*10 ³	0
CFU# present per mL culture after 15 days incubation	2.8*10 ³	0	NA	NA	NA	NA	NA	NR	NA
CFU# present per mL culture after 45 days incubation	NR	NA	NA	NA	NA	NA	NA	0	NA
CFU# present per mL culture after 60 days incubation	3.7*10 ²	NA	NA	NA	NA	NA	NA	NA	NA
CFU# present per mL culture after 80 days incubation	4.4*10 ¹	NA	NA	NA	NA	NA	NA	NA	NA
CFU# present per mL culture after 100 days incubation	0	NA	NA	NA	NA	NA	NA	NA	NA

*While all isolates were maintained, and tested for anaerobic growth, in their respective isolation-media, *Methylophaga* was maintained/tested in ASW-methanol (ASWM).
MSTYP = MSTY medium supplemented with pyruvate; ASWTYP = ASWTY medium supplemented with pyruvate; ASWMF = ASWM medium supplemented with fructose; full-forms of all the other abbreviated media names are available in the text. NA = Not applicable; NR = Not recorded.

‡All media-types used to check anaerobic growth/survival were supplemented with MnO₂, Na₂SO₄, NaNO₃, Fe₂O₃, (CH₃)₂SO and (CH₃)₃NO.

#CFU or colony forming units present were counted by taking out 1 mL of the liquid culture, then plating individual dilution grades onto triplicate agar plates of the corresponding media and incubating the plates aerobically. Colony-counts in the different dilution-plates (checked until no further colony appeared) were multiplied by corresponding dilution factors, then summed-up across all the plates and finally averaged to get the number of CFU present per mL of the liquid culture.

cytochrome c oxidase (*coxABCD*), *ccb₃*-type cytochrome c oxidase (*ccoNOPQ*) and/or cytochrome-*bd* ubiquinol oxidase (*cydABX* and *appX*) (Table 2 and Table S3, Supporting Information). A total of 19 out of the 25 contig-collections contained genes for aerobic oxidation of methane/ammonia/alcohols [these included genes encoding soluble methane monooxygenase (*mmoXYZBCD*) and methanol dehydrogenase (*mxoA*)] (Table S4, Supporting Information), whereas all 25 contained genes for the aerobic oxidation of thiosulfate (*soxC* for sulfane dehydrogenase), sulfite (SUOX for sulfite:acceptor oxidoreductase) and/or various organosulfur compounds (*dmoA* for dimethyl-sulfide monooxygenase, *ssuD* for alkanesulfonate monooxygenase, *sfnG* for dimethylsulfone monooxygenase and *mtoX* for methanethiol oxidase) (Table S5, Supporting Information).

Concurrent with the metagenomic data, metatranscriptome analysis for the microbial community living at the deepest sediment-sample (275 cmbsf) of SSK42/6 revealed sequences corresponding to aerobic-metabolism-related genes. The assembled and annotated metatranscriptomic sequence data (mRNA transcript catalog) of this sample encompassed diverse respiratory oxidase enzymes such as cytochrome *bd*-type quinol oxidase, cytochrome *ccb₃*-type oxidase, cytochrome c oxidase. In addition, diverse other oxidases such as aldehyde oxidase, amine oxidase, coproporphyrinogen III oxidase, FAD linked oxidase, glycine oxidase, glycolate oxidase, hydroxyglutarate oxidase, l-aspartate oxidase, protoporphyrinogen oxidase, sulfite oxidase (and its variant sulfur dehydrogenase that oxidizes the sulfane sulfur of thiosulfate), thiol-disulfide oxidase and ubiquinol oxidase were also detected (Table S6, Supporting Information).

Live aerobic bacteria are ubiquitous in the ASOMZ sediments

Most probable number of live, obligately or facultatively aerobic, chemoorganoheterotrophs and sulfur-chemolithoautotrophs (Tables S7-S10, Supporting Information) were found to be high along both SSK42/5 (up to 1.1×10^5 and 1.6×10^3 cells/g sediment, at individual sample-sites, respectively) and SSK42/6 (up to 1.1×10^7 cells/g sediment, at individual sample-sites, for both chemoorganoheterotrophs and chemolithoautotrophs). Simultaneously, from the 275 cmbsf sediment-sample of SSK42/6, aerobic microbial consortia could be successfully enriched via fully oxic incubation in chemolithoautotrophic ASWT, and mixotrophic MSTY and ASWTY, broths. A total of 27 bacterial strains were isolated from these enrichment cultures. Taxonomically, the isolates formed nine species-level clusters, of which seven were classified under distinct genera (namely, *Cereibacter*, *Guyarkeria*, *Methylophaga*, *Pseudomonas*, *Pusillimonas*, *Stenotrophomonas* and *Sulfitobacter*) while two clusters isolated separately in MSTY and ASWTY represented two distinct species of *Halomonas* (Table 1).

One representative strain from each cluster was tested for anaerobic growth in its specific medium supplemented with the electron acceptors NaNO_3 , Fe_2O_3 , MnO_2 , Na_2SO_4 , $(\text{CH}_3)_2\text{SO}$ and $(\text{CH}_3)_3\text{NO}$, which are all known to act as respiratory substrates in diverse marine microorganisms (López and Duarte 2004; Jørgensen and Kasten 2006; Lidbury, Murrell and Chen 2014). Subsequently, all the representative strains were also tested for anaerobic growth in their specific media supplemented with each of the above six electron acceptors separately. Furthermore, each of the current isolates was tested for anaerobic growth in their specific media supplemented with humic acids, or humic

acids and Fe_2O_3 in combination. Aerobically incubated versions of all the above cultures exhibited cellular growth yields comparable with those obtained during aerobic growth in the same media-types minus any anaerobic respiratory substrate (Table S11, Supporting Information).

After 10 days of anaerobic incubation in their specific media supplemented with NaNO_3 , Fe_2O_3 , MnO_2 , Na_2SO_4 , $(\text{CH}_3)_2\text{SO}$ and $(\text{CH}_3)_3\text{NO}$, the representative strains of the MSTY-isolated-*Halomonas* (SBBP1), *Pusillimonas ginsengisoli* (MTCC12559) and *Stenotrophomonas* (SBPC3) clusters exhibited growth (Table 1). These three strains could also grow anaerobically in their respective media when NaNO_3 , but not the other five compounds, were provided individually as respiratory substrate or terminal electron acceptor (Table S12, Supporting Information). In contrast, after 10 days of anaerobic incubation in their specific media containing the six-electron-acceptor mixture, the representative *Guyarkeria* strain (SB14A) retained only 4.6% of the initial cell count whereas the representative strains of the *Cereibacter changlaensis* (MTCC12557), ASWTY-isolated-*Halomonas* (MCC3301), *Methylophaga* (MTCC12599), *Pseudomonas bauzanensis* (MTCC12600) and *Sulfitobacter* (MCC3606) clusters had no viable cells left (Table 1). The use of the six individual electron acceptors separately in six different culture sets also did not help any of these strains to grow anaerobically (Table S12, Supporting Information). After 10 days of anaerobic incubation in their specific media supplemented with humic acids, or humic acids and Fe_2O_3 in combination, none of the new isolates had any viable cell left in the culture (Table S12, Supporting Information).

When the *C. changlaensis*, ASWTY-isolated-*Halomonas*, *P. bauzanensis* and *Sulfitobacter* strains were tested for fermentative growth/survival in their specified media supplemented with sodium pyruvate, none, except the *Pseudomonas* strain, had any viable cell left after 5 days of anaerobic incubation. The *Pseudomonas* strain too had no viable cell left after 45 days. Fermentative survival of the *Methylophaga* strain was tested in ASW-methanol-fructose (Janvier et al. 1985), and that of the *Guyarkeria* strain in ASWT [since no strain of *Guyarkeria* (Boden 2017), including SB14A, can utilize extraneous organic carbon, this experiment was designed to test survival via fermentation of stored polyglucose (Beudeker, Boer and Kuenen 1981)]—the two strains had no viable cells left after 15 and 100 days respectively (Table 1).

Aerobic sulfur-chemolithotrophy in the new isolates

O_2 -dependent sulfur-chemolithotrophic attributes were tested in the representative strains of the nine species-level clusters (details given in Supplementary Results). Of these, the facultatively anaerobic *Halomonas* SBBP1, albeit isolated in MSTY, was found to be a chemoorganoheterotroph; *P. ginsengisoli* MTCC12559 was a thiosulfate- and tetrathionate-oxidizing chemolithoheterotroph [phenotypes consistent with the pathways delineated for another *P. ginsengisoli* isolate from the same 275 cmbsf sediment-sample of SSK42/6 (Mandal et al. 2020b)]; and *Stenotrophomonas* SBPC3 could oxidize only thiosulfate to tetrathionate in chemolithoautotrophic medium.

Of the six obligately aerobic strains, *C. changlaensis* MTCC12557 and *Sulfitobacter* MCC3606 could oxidize only thiosulfate directly to sulfate, while *Halomonas* MCC3301 and *P. bauzanensis* MTCC12600 could oxidize only thiosulfate to tetrathionate, in chemolithoautotrophic medium; *Guyarkeria* SB14A, however, oxidized both thiosulfate and tetrathionate

Table 2. Total number of homologs identified for the various structural genes¹ associated with the different mechanisms of aerobic respiration, within the metagenome assemblies obtained for the individual sediment-samples of SSK42/5 and SSK42/6.

Sediment-core	Sediment-depth explored (in cmbsf)	Mechanism of aerobic respiration identified (KEGG metabolic module)			
		Aerobic respiration by Cytochrome c oxidase, <i>aa3</i> -type (M00154)	Aerobic respiration by Cytochrome c oxidase, prokaryotes (M00155)	Aerobic respiration by Cytochrome c oxidase, <i>cbb3</i> -type (M00156)	Aerobic respiration by Cytochrome-bd ubiquinol oxidase (M00153)
SSK42/5	0	15	86	31	6
	15	16	64	40	5
	45	17	55	37	3
	60	17	66	55	3
	90	25	99	60	4
	120	23	84	62	2
	140	9	30	21	2
	160	25	95	71	1
	190	18	82	51	5
	220	13	50	45	0
	260	15	64	44	5
	295	16	84	66	4
	SSK42/6	2	27	96	81
30		22	97	71	14
45		37	126	101	19
60		36	110	123	25
75		37	142	119	15
90		26	72	78	16
120		27	82	91	24
135		18	64	66	10
175		17	80	80	36
220		3	3	6	0
250		1	2	5	1
265		3	24	13	8
275		38	140	110	58

¹Table S3 (Supporting Information) lists of the various structural genes associated with the different mechanisms of aerobic respiration and enumerates the homologs identified under each genes, within the metagenome assemblies obtained for the individual sediment-samples of SSK42/5 and SSK42/6.

directly to sulfate without forming any detectable intermediate [phenotype consistent with the characteristics of other *Gyuparkeria* strains (Boden 2017)]. *Methylophaga* MTCC12599, only in the presence of methanol, oxidized portions of the supplied thiosulfate to tetrathionate and sulfate, even as no tetrathionate was oxidized. Moreover, MTCC12599, unlike other well-studied *Methylophaga* (Boden et al. 2010, 2012) did not oxidize dimethylsulfide.

Whole genome sequence analysis of the obligately aerobic isolates

Whole genome sequencing, assembly and annotation were carried out for the six obligately aerobic strains *C. changlaensis* MTCC12557, *Gyuparkeria* sp. SB14A, *Halomonas* sp. MCC3301, *Methylophaga* sp. MTCC12599, *P. bauzanensis* MTCC12600 and *Sulfitobacter* sp. MCC3606 (GenBank accession numbers given in Table 1; basic characteristics of the genomes described in Supplementary Results and Table S13, Supporting Information). While the sulfur-oxidation-related gene contents of the six strains corroborated their chemolithotrophic phenotypes (Fig. 2; also see Supplementary Results), their genomes also contained several oxidase-encoding genes, including those for aerobic respiration by *cbb3*-type cytochrome c oxidase. All except *Methylophaga* sp. MTCC12599 contained *aa3*-type cytochrome c oxidase genes, while all except *Gyuparkeria* sp. SB14A had genes

for cytochrome-*bd* ubiquinol oxidase (see Tables S14–S19, Supporting Information, in the alphabetical order of the six isolates). Notably, however, no genetic system for respiration using nitrate, ferric, manganic or sulfate ion, or dimethyl sulfoxide, or trimethylamine N-oxide, was detected when the six annotated genomes were scrutinized manually as well as by using the web-based tool *KEGG Mapper—Reconstruct Pathway* (https://www.genome.jp/kegg/tool/map_pathway.html). Furthermore, none of the large number of genes that are known to be associated with extracellular electron transfer (He et al. 2019) and anaerobic glycolysis (Hong and Gu 2009; Buckel and Thaur 2013) were detected upon manual and *KEGG Mapper—Reconstruct Pathway*-based scrutiny of the genomes.

The obligately aerobic isolates are widespread across the ASOMZ sediment-horizons explored

Ubiquitous prevalence of the six obligately aerobic strains across the ASOMZ sediment-horizons was evident when substantial proportions of metagenomic reads obtained from the distinct sediment-samples of SSK42/5 and SSK42/6 mapped onto each of the six newly-sequenced genomes (Fig. 3; Table S13, Supporting Information). In SSK42/5 and SSK42/6, approximately 0.01–0.3% and 0.04–19.05% metagenomic reads from the individual sample-sites matched with sequences from the six different genomes respectively; the only ‘metagenome-genome’ pair to have no matching sequence was ‘45 cmbsf of SSK42/6 and

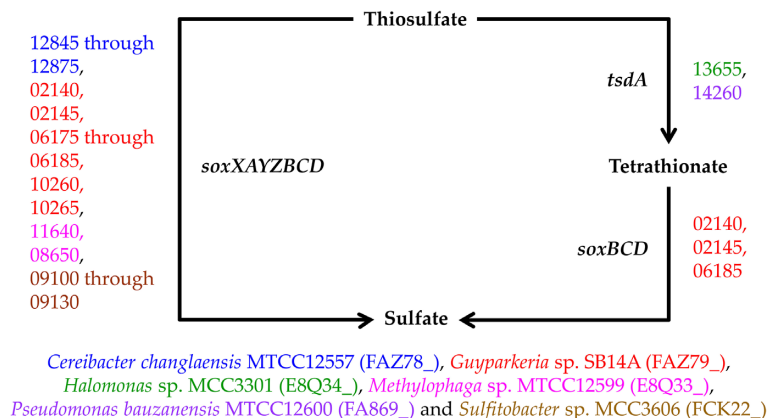


Figure 2. Overview of the different sulfur oxidation processes and genes identified in the six obligately aerobic strains isolated from the 275 cmbsf sediment-sample of SSK42/6. The sulfur-oxidation-related genes (Ghosh and Dam 2009) identified in the 91.58–99.84% complete genomes of *Cereibacter changlaensis* MTCC12557, *Guyparkeria* sp. SB14A, *Halomonas* sp. MCC3301, *Methylophaga* sp. MTCC12599, *Pseudomonas bauzanensis* MTCC12600 and *Sulfitobacter* sp. MCC3606 were consistent with their chemolithotrophic phenotypes, but within the 98.01% complete genome of *Methylophaga* sp. MTCC12599, which produced both tetrathionate and sulfate from thiosulfate, only *soxY* and *soxZ* genes were identified—these two genes neither have any documented involvement in thiosulfate to tetrathionate oxidation nor can oxidize thiosulfate to sulfate without *soxXABCD* (Ghosh and Dam 2009); also within the 95.9% complete genome of *Guyparkeria* sp. SB14A no *tetH* or *thdT* gene was identified for tetrathionate oxidation even as *soxB**CD* are known to be involved in this process of other bacteria (Pyne et al. 2018).

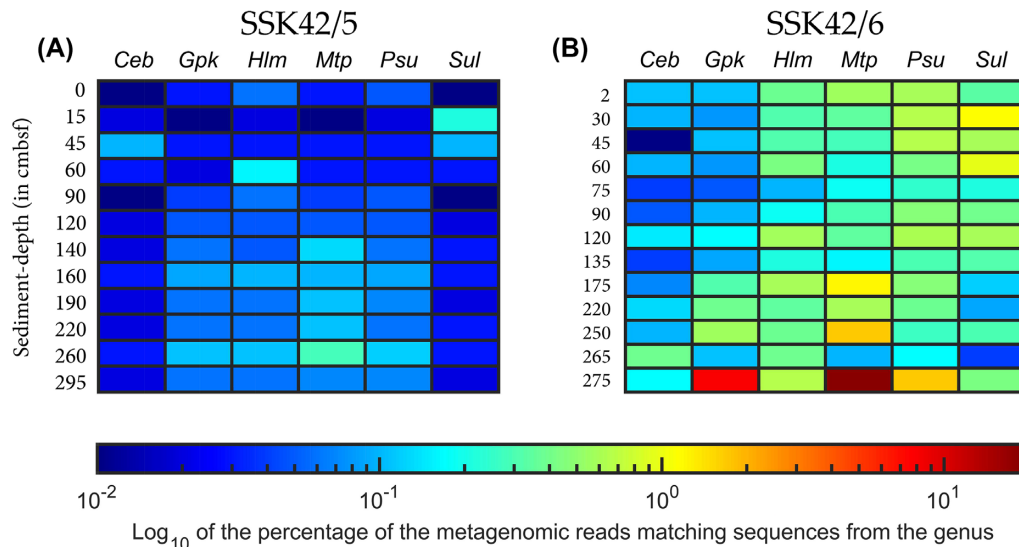


Figure 3. Heat map comparing the percentages of metagenomic reads from individual sediment-samples of (A) SSK42/5 and (B) SSK42/6 that matched with genomic sequences of the six obligately aerobic bacterial isolates: (Ceb) *Cereibacter changlaensis* MTCC12557, (Gpk) *Guyparkeria* sp. SB14A, (Hlm) *Halomonas* sp. MCC3301, (Mtp) *Methylophaga* sp. MTCC12599, (Psu) *Pseudomonas bauzanensis* MTCC12600 and (Sul) *Sulfitobacter* sp. MCC3606. For each percentage value, its Log_{10} has been plotted in the z-axis of the heat map, so as to resolve the wide span of the data. Percentage level of matched reads for individual pairs of metagenomic-genomic datasets ranged between 0 and 19.05, with intermediate values in the order of 10^{-2} to 10^1 . Since 0 is less than any minimum value possible in the log scale, the deepest blue square of panel b, which apparently matches the order of 10^{-2} in the color scale, actually corresponds to the real value 0 (see Table S13, Supporting Information).

C. changlaensis MTCC12557'. Prevalence of reads matching the six new isolates was relatively higher for the metagenomes of SSK42/6, and then within this core, highest for 275 cmbsf, the sample-site from where the strains were isolated.

The obligately aerobic strains are metabolically active, and most probably growing, in-situ

Correspondence between genomic sequences of the obligately aerobic isolates and metatranscriptomic sequences from their habitat illustrated that the strains performed active aerobic metabolism, cell division and growth in-situ. In these

experiments, all paired-end mRNA reads present in the rRNA-sequence-free metatranscriptomic dataset obtained for the 275 cmbsf sample of SSK42/6 were mapped onto different manually-curated gene-catalogs of the individual obligately aerobic isolates. With respect to the aerobic-metabolism-related gene-catalogs of the individual strains, maximum number of metatranscriptomic read-pairs corresponded to *Guyparkeria* SB14A, with 0.03% of the total 26 496 769 read-pairs examined matching concordantly with genes for *aa₃*-type cytochrome c oxidase, *cbb₃*-type cytochrome oxidase and various other oxidase enzymes that are all concerned with the catalysis of redox reactions involving O_2 as the electron acceptor (Fig. 4; Table S20, Supporting Information). For *C. changlaensis* MTCC12557

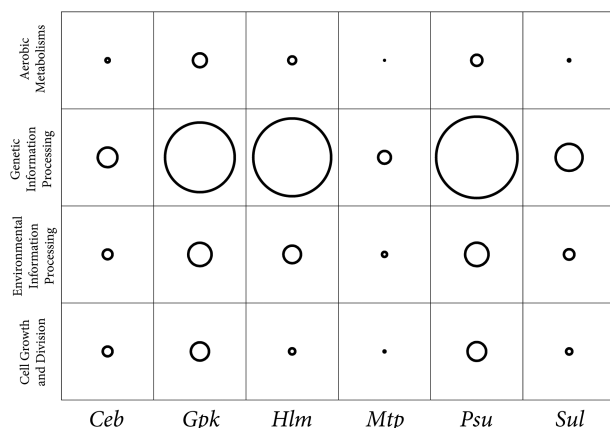


Figure 4. Bubble plot comparing the percentages of metatranscriptomic read-pairs from 275 cbsf of SSK42/6 which matched concordantly with genes belonging to different metabolic categories in the six obligately aerobic strains isolated from the same sediment-sample: (*Ceb*) *Cereibacter changlaensis* MTCC12557, (*Gpk*) *Guyarkeria* sp. SB14A, (*Hlm*) *Halomonas* sp. MCC3301, (*Mtp*) *Methylophaga* sp. MTCC12599, (*Psu*) *Pseudomonas bauzanensis* MTCC12600 and (*Sul*) *Sulfitobacter* sp. MCC3606. Size of the circles is proportional to the percentage of metatranscriptomic read-pairs matched. The single series bubble plot was constructed using the Scatter tool in MATLAB 2017b (Martinez, Martinez and Solka 2017).

(Table S21, Supporting Information), *Halomonas* MCC3301 (Table S22, Supporting Information), *P. bauzanensis* MTCC12600 (Table S23, Supporting Information) and *Sulfitobacter* MCC3606 (Table S24, Supporting Information) respectively, 0.003, 0.009, 0.02 and 0.0007% of the total 26 496 769 metatranscriptomic read-pairs examined matched concordantly with their genes for *aa₃*-type cytochrome *c* oxidase, *cbb₃*-type cytochrome oxidase, cytochrome-*bd* ubiquinol oxidase and various other oxidases (Fig. 4). For *Methylophaga* MTCC12599, only 0.00005% of the total 26 496 769 read-pairs tested matched concordantly with its gene for cytochrome-*bd* ubiquinol oxidase subunit II (Fig. 4; Table S25, Supporting Information).

All paired-end mRNA reads of the metatranscriptomic dataset were also mapped onto the obligately aerobic isolates' gene-catalogs pertaining to the core metabolic categories (i) Genetic Information Processing (*Replication and repair*: DNA replication; *Transcription*: RNA polymerase; *Translation*: Aminoacyl-tRNA biosynthesis and Ribosome), (ii) Environmental Information Processing (*Membrane transport*: ABC transporters, Bacterial secretion system and Phosphotransferase system) and (iii) Cell Growth and Division (Cell cycle), abbreviated hereafter as GEC gene-catalogs. *Guyarkeria* SB14A had the maximum (0.84) percentage of the total 26 496 769 read-pairs tested matching concordantly with 181 genes of its 183-gene strong GEC catalog (Fig. 4; Table S26, Supporting Information). For *C. changlaensis* MTCC12557 (Table S27, Supporting Information), *Halomonas* MCC3301 (Table S28, Supporting Information), *P. bauzanensis* MTCC12600 (Table S29, Supporting Information) and *Sulfitobacter* MCC3606 (Table S30, Supporting Information), 0.09, 0.94, 1.10 and 0.13% of the total metatranscriptomic read-pairs examined matched concordantly with 245, 132, 147 and 133 genes of their 397-, 273-, 252- and 310-gene strong GEC catalogs respectively (Fig. 4). For *Methylophaga* MTCC12599, only 0.03% of the total read-pairs examined matched concordantly with 34 genes of its 193-gene strong GEC catalog (Fig. 4; Table S31, Supporting Information).

DISCUSSION

Biogeochemical implications of aerobic bacteria in OMZ sediments

In marine territories near the continental margins, where productivity and organic matter deposition are high, aerobic bacteria predominate in the water-columns and sea-floors but rarely down the sediment-depths with depleting dissolved O_2 in the pore-fluids. This population dynamics may directly or indirectly depend on *in-situ* sedimentation rate, bottom-water O_2 concentration, O_2 -diffusion coefficient, activities of sediment-burrowing animals and the nature of the organic matter deposited, (Canfield 1994; Kristensen, Ahmed and Devol 1995; Jørgensen 2006; Burdige 2007; Middelburg 2019). Furthermore, within the shelf realm, sediment systems underlying hypoxic water-columns are epitomized by copious flux of labile (yet complex) organic matter across the sea-bed (Cavan et al. 2017; Jessen et al. 2017) because low dissolved O_2 apparently limits their breakdown prior to deposition (Revsbech et al. 2009; Ulloa et al. 2012; Garcia-Robledo et al. 2017). Concurrently, it is remarkable that organic-carbon remineralization and simple-fatty-acids-requiring anaerobic microbial processes of the carbon-sulfur cycle are also highly active in these sediment regimes (van der Weijden, Reichart and Visser 1999; Schulte, Mangelsdorf and Rullkötter 2000; Seiter, Hensen and Zabel 2005; Bowles et al. 2014; Cowie et al. 2014; Fernandes et al. 2018).

In the ~3 m ASOMZ sediment-horizons explored in SSK42/5 and SSK42/6, near-complete depletion of the total organic carbon (TOC) content has been observed within the core-lengths, in tandem with the considerable prevalence of simple-fatty-acids-requiring microorganisms until the core-bottoms (Fernandes et al. 2018). These features indicate that within these sediment-horizons, effective depolymerization/hydrolysis occurs not only for the labile components of the TOC (for example, proteins and carbohydrates from marine biomass) that are degraded (to water-soluble simple fatty acids) with equal efficacy in the presence and absence of O_2 (Cowie, Hedges and Calvert 1992; Prahl et al. 1997; Aller and Blair 2004), but also the potential refractory fractions which warrant the mediation of aerobic microbial catabolism (Kristensen, Ahmed and Devol 1995; Burdige 2007). In this scenario, the metabolically active and potentially growing aerobic bacterial communities revealed across the sediment-horizons provide the necessary mechanistic baseline that was thus far missing in the ecological/biogeochemical framework of the OMZ sediment system. Likewise, scarcity of dissolved O_2 down the OMZ sediment-depths is also expected to limit chemolithotrophic oxidation of reduced inorganic compounds, since most of the biochemical pathways known for such processes are mechanistically aerobic (Ghosh and Dam 2009; Emerson, Fleming and McBeth 2010; Kuypers, Marchant and Kartal 2018). In this context, the aerobic sulfur-chemolithotrophic microflora revealed illustrates the possibilities of sulfide back-flux and reconversion to sulfate (including potential pyrite dissolution leading to metal mobilization) within the sediment system.

Potential source of O_2 for aerobic microorganisms in ASOMZ sediments

Albeit technical limitations disallowed O_2 measurement in SSK42 sediment-cores, questions remain as to how aerobic microorganisms could be abundant and active in these highly-reduced (H_2S -containing) environments (Fernandes et al. 2018)

where there is little scope of O₂ influx. Metagenomic, metatranscriptomic and co-culture-based data, however, suggested that potentially-symbiotic perchlorate-/chlorate-reducing microorganisms could be sustaining the aerobic microflora *in-situ*, via cryptic O₂ supply-consumption partnership similar to the one reported for picocyanobacteria and nitrite-oxidizers in the acutely hypoxic waters of eastern tropical North and South Pacific (Garcia-Robledo et al. 2017). For instance, (per)chlorate reductase and chlorite dismutase genes were identified in the assembled metagenomes of almost all the sediment-samples investigated (Table S32, Supporting Information); perchlorate-respiring microbial consortia (enriched from different SSK42-samples) were found to help the obligately aerobic *Guypparkeria* SB14A grow in anaerobic co-cultures (Supplementary Results); (per)chlorate reductase homologs were also there in the assembled metatranscriptome of the 275 cmbsf sediment-sample from SSK42/6 (Table S33, Supporting Information). No perchlorate, however, could be detected in the pore-waters of SSK42-samples using an ion chromatographic technique which had a 10 μM detection-limit (Supplementary Methods). Notably, perchlorate-/chlorate-reduction is taxonomically/ecologically widespread and evolutionarily ancient, but no habitat of perchlorate-/chlorate-reducers has thus far been reported for detectable chlorine oxyanions, plausibly because these substances accumulate to easily-detectable levels only in environments that are extremely-poor in microbial diversity/activity; elsewhere they are readily reduced by the *in-situ* microflora and therefore remain undetectable (Rajagopalan et al. 2006; Catling et al. 2010; Kounaves et al. 2010; Rao et al. 2010; Liebensteiner, Oosterkamp and Stams 2016). So far as the perchlorate-/chlorate-reducers of ASOMZ sediments are concerned, reliance on this respiration mechanism alongside close association with aerobic microorganisms seems to be mandatory for their survival. Perchlorate-/chlorate-reducers generally respire O₂ and NO₃ in preference over chlorine oxyanions, and for some of them 2–6 mg/L O₂ inhibits perchlorate reduction irreversibly (Bardiya and Bae 2011). However, in redox-stratified and apparently unbioturbated sediments as those of SSK42/5 and SSK42/6 (Fernandes et al. 2018), O₂ is consumed within a few cmbsf and nitrate may penetrate slightly deeper than that; so perchlorate-respiration, and therefore an assurance for low-O₂ micro-environment (via symbiosis with O₂-scavenging aerobes) seems indispensable for the *in-situ* survival of perchlorate-/chlorate-reducers.

More investigations of sedimentary microbiology and geochemistry, focused on aerobic communities and pathways of cryptic aerobiosis, are required across marine OMZs to comprehensively comprehend the ecological scopes/significances of aerobic life in acutely-O₂-limited environments.

AUTHOR CONTRIBUTIONS

W.G. conceived the study, designed the experiments, interpreted the results and wrote the paper. S.B. performed all the experiments, harnessed all the modules of the study and analyzed and curated the data. A.M. led the SSK42 mission and all geochemical studies therein. R.C. and A.M. interpreted the results and made intellectual contributions to the work and the manuscript. C.R., J.S., M.J.R., T.M., M.A., S.F. and A.P. performed bioinformatic studies and/or data analyses. S.M., J.S., N.M., S.C., P.P., P.K.H. and R.R. performed microbiological experiments. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

We express gratitude to the Director of CSIR-National Institute of Oceanography for making the research cruise SSK42 happen. Support extended by the CSIR-NIO Ship Cell and crew members of SSK42 is recognized gratefully. S.B. and M.A. got fellowship from Bose Institute. C.R. and M.J.R. were endowed with fellowships by University Grants Commission, Government of India (GoI). SM received a fellowship from Department of Science and Technology, GoI. J.S., P.P., R.R. and S.F. obtained their fellowships from Council of Scientific and Industrial Research, GoI. N.M. got fellowship from Science and Engineering Research Board, GoI. S.C. received a fellowship from the Department of Biotechnology, GoI.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSLE](https://www.femsle.com) online.

FUNDING

The microbiological studies were funded by Bose Institute (via Intramural Faculty Grants) and Earth System Science Organization, Ministry of Earth Sciences (MoES), GoI via the extramural grant MoES/36/00IS/Extra/19/2013; the research cruise SSK42 was also funded by MoES (GAP2303).

Conflicts of interest. None declared.

DATA AVAILABILITY

All nucleotide sequence data have been deposited in NCBI Sequence Read Archive (SRA) or GenBank under the BioProject accession number PRJNA309469: (i) the whole metagenome shotgun sequence datasets have the Run accession numbers SRR3646127 through SRR3646132, SRR3646144, SRR3646145, SRR3646147, SRR3646148, SRR3646150 through SRR3646153, SRR3646155 through SRR3646158, SRR3646160 through SRR3646165; SRR3570036, SRR3570038, SRR3577067, SRR3577068, SRR3577070, SRR3577071, SRR3577073, SRR3577076, SRR3577078, SRR3577079, SRR3577081, SRR3577082, SRR3577086, SRR3577087, SRR3577090, SRR3577311, SRR3577337, SRR3577338, SRR3577341, SRR3577343, SRR3577344, SRR3577345, SRR3577347, SRR3577349, SRR3577350, SRR3577351; (ii) the whole genome sequences have the GenBank accession numbers SWAW01000000, SSXS01000000, SZNL01000000, SSXT01000000, SWAV01000000 and SWAU01000000; (iii) the whole metatranscriptome sequence dataset has the Run accession number SRR7991972.

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