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BRIEF COMMUNICATION

"*Candidatus* Macondimonas diazotrophica", a novel gammaproteobacterial genus dominating crude-oil-contaminated coastal sediments

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Abstract

Modeling crude-oil biodegradation in sediments remains a challenge due in part to the lack of appropriate model organisms. Here we report the metagenome-guided isolation of a novel organism that represents a phylogenetically narrow (>97% 16S rRNA gene identity) group of previously uncharacterized, crude-oil degraders. Analysis of available sequence data showed that these organisms are highly abundant in oiled sediments of coastal marine ecosystems across the world, often comprising ~30% of the total community, and virtually absent in pristine sediments or seawater. The isolate genome encodes functional nitrogen fixation and hydrocarbon degradation genes together with putative genes for biosurfactant production that apparently facilitate growth in the typically nitrogen-limited, oiled environment. Comparisons to available genomes revealed that this isolate represents a novel genus within the *Gammaproteobacteria*, for which we propose the provisional name "*Candidatus* Macondimonas diazotrophica" gen. nov., sp. nov. "*Ca*. M. diazotrophica" appears to play a key ecological role in the response to oil spills around the globe and could be a promising model organism for studying ecophysiological responses to oil spills.

Crude-oil-impacted shorelines: an understudied ecological niche

The Deepwater Horizon (DWH) oil spill released over 780 million liters of oil and large amounts of natural gas (~1.7×10^{11} g) into the Gulf of Mexico and consequently, had a widespread impact on the pelagic, benthic, and coastal ecosystems [1, 2, 3]. While most studies have focused on the fate of the oil in the deep sea plume and sediments [4–6], the impact of the DWH spill on coastal marine ecosystems remains comparatively understudied [7]. Following the spill, large amounts of weathered oil contaminated an estimated 1773 km of the shoreline [8]. Long-term effects of the DWH spill are still not well understood owing to the stochasticity and complexity of ecosystem processes, as well as the lack of appropriate model microorganisms for studying the fate of oil in beach sands [3, 8, 9].

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The ubiquitous but uncharacterized *Gammaproteobacteria* in oiled sediments

Our previous studies revealed certain uncharacterized *Gammaproteobacteria* affiliated with the *Ectothiorhodospiraceae* family that showed high relative abundance in oil-contaminated sediments, exceeding the abundance of known hydrocarbon-degrading taxa (e.g., *Alcanivorax*, *Marinobacter*), especially during mid-to-late stages of degradation [7, 10, 11]. However, no cultivated members are available from this abundant *Gammaproteobacteria* group and thus, their physiology remains unknown. In these studies, we also leveraged a metagenome time series to characterize the microbial community response to the DWH oil perturbation in beach sands (Pensacola Municipal Beach, FL). Our work revealed the succession patterns of individual microbial populations that responded to the spill up to one year after oiling when petroleum hydrocarbons were no longer detectable above baseline. Contrary to our expectations, we observed that generalist taxa, as opposed to specialists, were favored by the perturbation [10]. Furthermore, PCR amplicon analysis of the nitrogen fixing genes (*nifH* gene) from these sands showed an increased abundance of *nifH* genes associated with various uncharacterized *Gammaproteobacteria* in the oil-contaminated samples and returning to very low levels in the recovered sands (Gaby et al., unpublished). This was a potentially important finding since oil biodegradation is often nitrogen-limited, as exemplified by the addition of nitrogen fertilizer during cleanup efforts for the Exxon Valdez spill in Prince William Sound, Alaska [12]. A particular allele of *nifH* showed much higher abundance than the rest. In order to identify the full genomic context of this *nifH* gene and exact phylogenetic affiliation, targeted population reconstruction using visual inspection of the read coverage patterns of the assembly (Supplemental methods) yielded a draft metagenome-assembled genome (MAG-01) that included the abundant *nifH* gene allele. MAG-01's abundance increased from below detection levels in the clean/pre-spill beach sand samples to ~30% of the entire microbial community in oiled samples, returning to low abundance levels in the recovered sediments (Fig. 1, Suppl. Fig. S1).

Targeted isolation efforts and metabolic versatility of the recovered isolate

Functional annotation of MAG-01 revealed putative genes for hydrocarbon degradation, nitrogen fixation, methanotrophy, urea metabolism, biosurfactant production, nutrient scavenging, and other related processes that could enhance growth in oil-contaminated environments (Suppl. Figs. S2, S3, Suppl. Table S3). Mass transfer limitations, nutrient (mainly nitrogen) and oxygen availability largely dictate the fate of the buried hydrocarbons and their bioavailability for microbial remediation [7, 13, 14]. Hence, the functions identified were likely important for successfully coping with the oil perturbation and resulting in the MAG-01 population dominating the oiled microbial communities. Read recruitment plots of the metagenomes revealed an even coverage of MAG-01, at high nucleotide identity (>98%), indicative of a sequence-discrete population (Fig. 1 and Suppl. Fig. S4) [15]. However, its 16S rRNA gene was not assembled, as is common in binning efforts [16], which prevented further taxonomic analysis. In an effort to identify the exact taxonomic affiliation of MAG-01 and further validate its genome sequence, visual inspection of the MAG-01 assembly, complemented with PCR walking for linking the rRNA operon, yielded a nearly complete genome.

To isolate the organism represented by the MAG-01 genome, enrichments were carried out using oiled sands collected at Pensacola beach as inocula in nitrogen-free minimal artificial seawater liquid media with no other added carbon source or nutrients. Liquid aerobic enrichments were incubated for nine weeks before plating on agar plates containing the same (solidified) media supplemented with 0.2% (w/v) of source Macondo/MC252 oil as the sole carbon and energy source. Through colony PCR screening with the primers used in PCR walking above that were specific to MAG-01, the isolate KTK-01 was recovered, showing 100% nucleotide identity to MAG-01's 16S rRNA gene (see Supplementary Online Methods for further details). The genome sequence of KTK-01 showed 99.8% genome-aggregate average nucleotide identity (ANI) to MAG-01, revealing that it was a member of the natural population represented by MAG-01. Furthermore, several of the bioinformatically predicted functions mentioned above such as hexadecane degradation and nitrogen fixation were experimentally verified. KTK-01's *nifH* was also overexpressed in laboratory mesocosm experiments containing beach sands with added Macondo oil when compared to the un-oiled controls (Karthikeyan et al., unpublished data), indicating that it was functional under oiled conditions.

Ecological pervasiveness of the 16S rRNA gene sequence of the isolate

Screening of publicly available 16S rRNA gene amplicon or clone library datasets revealed a remarkable distribution of identical or almost identical (>97% nucleotide identity) sequences to KTK-01 in hydrocarbon-contaminated sediments of coastal ecosystems across the globe (Fig. 2, Suppl. Fig. S4 and Suppl. Table S1). For instance, an operational taxonomic unit (OTU) identical to the 16S rRNA gene of KTK-01 (Fig. 2) and metagenomic reads covering ~350X

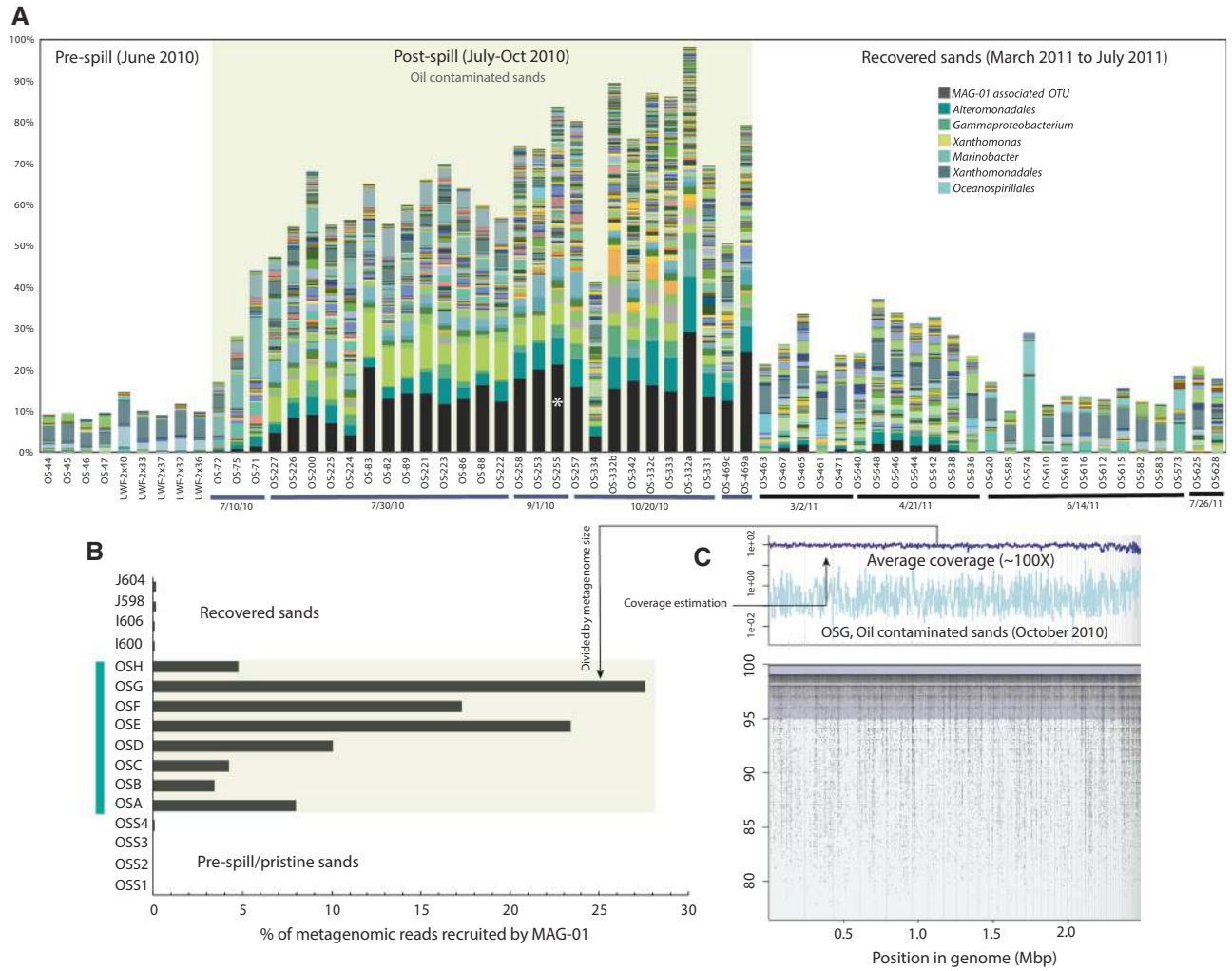

Fig. 1 Relative abundance of MAG-01 in oiled and clean beach sands from Pensacola beach, Florida (USA). **a** Abundance profiles of 16S rRNA gene-based OTUs detected in pre-oil, oiled and clean samples a year after the DWH oil spill. The MAG-01 16S-OTU is shown in black, at the bottom of the columns (denoted by an asterisk). Only the top 250 most abundant OTUs are shown. **b** Average coverage, representing relative abundance, of MAG-01 sequence (*x*-axis) by the reads of the metagenomic datasets described in [23] (*y*-axis). **c** (Bottom) Read recruitment plot showing where metagenomic reads of a contaminated sample (OS_G), which had the highest abundance of MAG-01, mapped (*x*-axis) and their identity (*y*-axis). (Top) The dark blue histogram represents coverage, i.e., how many times each nucleotide base is covered by reads on average, by reads matching the reference MAG-01 sequence, at ≥80 bp in length and ≥95% nucleotide identity, in 1000 bp-long windows; light blue represents reads matching at <95% identity. The evenness of the coverage of the genome on the metagenomic datasets shows a sequence-discrete population. Note that the coverage values shown in panel (**b**) are derived from the average coverage obtained in the recruitment plots (dark blue histogram, Panel (**c**)) after normalizing for the size of the metagenomic dataset, and that the MAG-01 is not detectable in pre-spill samples and has low abundance in metagenomes of recovered microbial communities

the KTK-01 genome (Suppl. Fig. S1) was also of the most dominant OTUs/population in other beach sands affected by the DWH spill in the States of Louisiana and Alabama. Furthermore, organisms with KTK-01-like sequences were among the dominant taxa responding to other major coastal oil spills, including the Prestige spill in the Galicia coast (>9% of total sequences) and in Cape Hallett in East Antarctica after an oil spill incident (Fig. 2, Suppl. Table S1). However, KTK-01-like sequences were below detection in the deep sea Macondo plume and oiled sediments, as well as in various uncontaminated water column metagenomes including those made available by the TARA Oceans expedition, underscoring an ecological niche specialization of KTK-01 in oiled, beach sands and coastal sediments.

Simultaneous hydrocarbon degradation and nitrogen fixation by a single organism is rather uncommon among isolated hydrocarbon-degrading bacteria whose genomes were available to bioinformatically assess functional gene content (0/16; Suppl. Table S2). Furthermore, these genomes recruited almost no reads from available

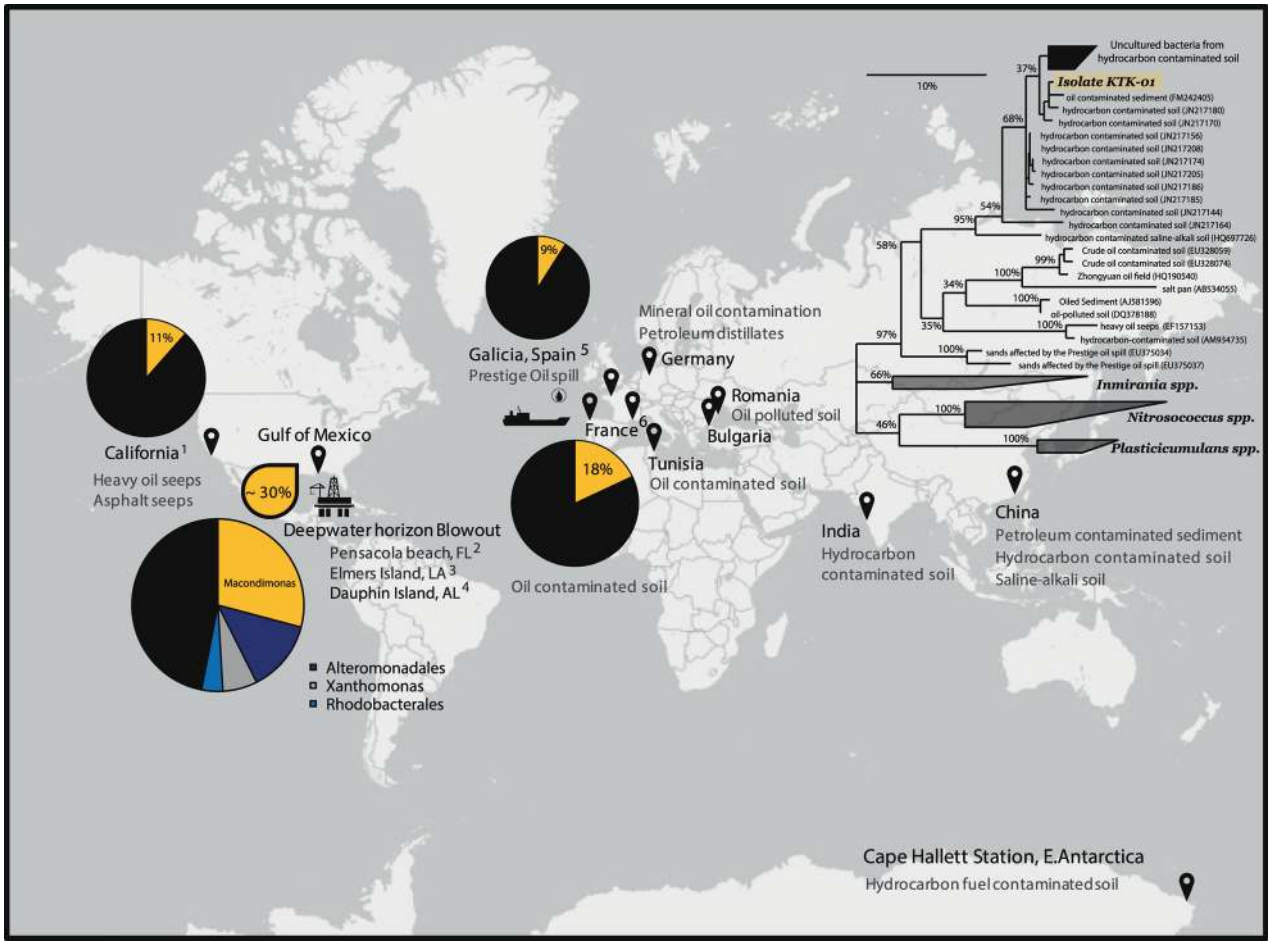

Fig. 2 Phylogeny and distribution of KTK-01-like 16S rRNA gene sequences in oil-contaminated sites across the globe. Pie charts represent the fraction of total sequences showing >97% nucleotide identity to the 16S rRNA gene sequence of KTK-01. For instance, 30% of the OTUs recovered from the beach sands impacted by Macondo oil matched the 16S rRNA gene sequence at this level. Accession numbers of the datasets used are provided in Supplementary Table S1. Inset: 16S rRNA gene phylogeny of KTK-01 and selected close relatives. Maximum likelihood, as implemented in RaxML and using all homologous positions of the bacterial alignment in the LTP_123 dataset, was used to obtain the phylogenetic tree shown. Bootstrap values are indicated next to the branches. Complete 16S rRNA phylogeny is shown in Suppl. Fig. S4

metagenomes of oiled coastal sediments, contrasting with the high abundance observed for KTK-01 (e.g., Fig. 1). Therefore, our data indicated that the common practice of providing a nitrogen source during enrichment efforts might have biased the known diversity of cultivated hydrocarbon-degraders, and that nitrogen fixation is likely a strongly selected trait during oil biodegradation in-situ.

Collectively, our results indicated that KTK-01 represents a highly promising model organism and a useful biomarker for the investigation of oil biodegradation in sediments, especially during mid-to-late phases of degradation (Fig. 1). In addition, the phylogenetically distant affiliation of KTK-01 with its closest relative (classified) species, together with distinct genomic (e.g., AAI value < 65%, which corresponds to the genus level [17]) and phenotypic traits (distinct diagnostic characters) indicated that the new isolate could be classified as a new genus and species. The closest classified relatives based on the Microbial Genomes Atlas (MiGA) webserver [18] were autotrophic sulfur oxidizing species, *Thioalkalivibrio sulfidiphilus* (48.32% AAI) and *Thiohalobacter thiocyanaticus* FOKN1 (47.81% AAI) that do not fix nitrogen or degrade higher alkanes like hexadecane (Table 1). *Thioalkalivibrio sulfidiphilus* HL-EbGr7 is also the closest classified relative by 16S rRNA gene identity (91.8%) (Suppl. Figs. S4-S6). For the new isolate we propose the name "*Candidatus* Macondimonas diazotrophica" gen. nov., sp. nov. Due to the relative slow growth of the isolate, which could delay effective validation of its name, and the high relevance of the findings reported here for crude oil biodegradation, the Candidatus option was used for strain KTK-01.

Table 1 Distinguishing characteristics of *Ca*. Macondimonas from its closest described genera

Characteristic	*Ca*. Macondimonas 1	*Inmirania* 2	*Thioalkalivibrio* 3	*Ectothiorhodospira* 4	*Nitrosococcus* 5	*Plasticicumulans* 6
Isolation source	Beach sands affected by Macondo oil	Thermal spring	Soda lakes	Salt lakes, soda lakes, estuaries	Seawater	Mixed culture biorecator
16S rRNA identity to str. KTK01	100	90.1	91.8	91.3	89.7	88.5
Nutritional group	COH	CLA	CLA	ANP	CLA	COH
Nitrogen fixation	+	−	−	+	−	−
Hexadecane oxidation	+	−	−	−	−	−
Phototrophic growth	−	−	−	+	−	−
Sulfide oxidation	−	−	+	+	−	ND
Temperature optimum, °C	22–30	65	30–35	25–40	25–30	30
NaCl range (% w/v)	1.46–3.0	0.5–3.5	1.2–10.5[a]	0–20		
NaCl optimum	1.92	1.5–2.0	2.3–11.7	0.5–8.0	2.45–3.5	
pH range	6.5–8.5	5.5–8.8	7.5–10.6	7.0–11.0		6.0–8.0
pH optimum	7.5	6.5	8.0–10.2	7.5–10.0	7.5–8.0	ND
Relationship to oxygen	Aerobe	Anaerobe[b]	Anaerobe	Anaerobe	Obligate aerobe	Obligate aerobe
DNA G+C content (%)	61.56	71.5	61.3–66.9	59.2–68.4	50.5–61.2	67.4

2: *Inmirania* [19], 3: *Thioalkalivibrio* [20], 4: *Ectothiorhodospira* [20], 5: *Nitrosococcus* [21], 6: *Plasticicumulans* [22]

COH Chemoorganoheterotroph, *CLA* Chemolithoautotroph, *ANP* anaerobic phototroph, *ND* Not Determined

[a]Up to 29.2 in some strains

[b]Microaerophile

Description of "*Candidatus* Macondimonas" gen. nov

Macondimonas, [Ma.con.di.mo'nas. L. fem. n. monas, a unit, a monad; N.L. fem. n. Macondimonas, a monad from Macondo Prospect, the site of DWH oil spill. Additionally, Macondo is a fictional town in *A Hundred Years of Solitude* by G. G. Márquez. In the book, the town of Macondo has a rapid population growth, a period of economic prosperity, and then a rapid population fall, which is reminiscent of the ecologic pattern observed for this group upon crude-oil exposure]. members of this genus exhibit a coccobacilli morphology and a heterotrophic aerobic metabolism. No phototrophic, nor chemoautotrophic growth, or their corresponding genes in the genome were observed. The type species is "*Ca*. Macondimonas diazotrophica".

Description of "*Candidatus* Macondimonas diazotrophica" sp. nov

"*Ca. M. diazotrophica*", [di.a.zo.tro'phi.ca. Gr. pref. *di*, in two; N.L. neut. n. *azotum* from Fr. n. *azote* (from Gr. prep. *a*, not; Gr. n. *zôê*, life; N.Gr. n. *azôê*, not sustaining life), nitrogen; N.L. pref. *diazo-*, pertaining to dinitrogen; Gr. adj. *trophikos -ê -on*, feeding, tending; N.L. fem. adj. *diazotrophica*, one that feeds on dinitrogen, named after its ability to fix atmospheric nitrogen] cells grown on solidified mineral artificial seawater media using hexadecane as substrate show a coccobacillus morphology, of about 0.6 µm in length and 0.35 µm in width, and formed circular colonies. Members of the species are aerobes, growing at a pH range of 6.5–8.5 with a pH optimum of 7.5, and a salinity range of 250–500 mM of NaCl, with an optimum concentration of 330 mM. The temperature range for optimal growth is 22–30 °C, with no growth observed at 4 °C and above 34 °C. Cells can grow with hexadecane and pyruvate as a sole carbon sources and fix nitrogen. Genome size is ~2.8 Mbp with a G+C% content of 61.56. The designated type material is strain KTK01, and its genome sequence can be found under NCBI BioSample accession number SAMN11302943.

Data availability

The data reported in this paper are publicly available through the Gulf of Mexico Research Initiative Information

& Data Cooperative (GRIIDC), under the accession numbers R5.x278.000:0014 and R5.x278.000:0002 (NCBI accession MH795143). The metagenome sequences as well as the assembled genome sequences are also available at http://enve-omics.ce.gatech.edu/data/.

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