# Denitrification genes in SAR11 and other ubiquitous lineages of marine bacteria isolated from the northern Benguela Upwelling System

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**Abstract**

Denitrification is a microbial process that leads to nitrogen loss from marine oxygen minimum zones (OMZs). The complete metabolic pathway for denitrification reduces nitrate to dinitrogen gas in four sequential steps. Many anaerobic and facultatively anaerobic bacteria are capable of the initial step of nitrate reduction to nitrite. Far fewer reduce nitrite to nitric oxide, nitrous oxide, or dinitrogen gas. In this study, we cultured and sequenced the complete genomes of 24 bacteria isolated from low dissolved oxygen waters (DO = 24 μM) in the northern Benguela Upwelling System (nBUS) OMZ to identify facultatively anaerobic bacteria with the genetic potential to contribute to denitrification. Most of the isolates obtained from the nBUS have denitrification genes (83%). They include several new species in the order *Pelagibacterales* (SAR11), as well as representatives from a previously uncultured family of *Arenicellales* (UBA868), a previously uncultured genus of *Paracoccaceae*, and a previously undescribed genus of *Porticoccaceae*. All ten nBUS SAR11 genomes encode a copper-containing nitrite reductase (*nirK*) and key components of a cbb3-type cytochrome c oxidase (*cco*), suggesting that they respire oxygen under low DO concentrations and have the potential to contribute to nitrogen loss by respiring nitrite to nitric oxide.

**Key words**

Marine, OMZ, Bacteria, Denitrification, SAR11, UBA868

38 **Significance Statement**

39 Vast genomic diversity has confounded sequencing efforts to identity the potential for
40 marine bacteria to contribute to denitrification in marine oxygen minimum zones (OMZs). To
41 identify facultatively anaerobic microbes with the genetic potential to contribute to marine
42 nitrogen loss, we cultured and sequenced the complete genomes of bacteria from low-oxygen
43 waters of the northern Benguela Upwelling System. Complete genomes allowed for a
44 comprehensive analysis of denitrification. Most bacterial isolates, including all ten SAR11, have
45 the genetic potential to contribute to denitrification. This suggests that some of the most
46 abundant lineages of marine bacteria in the oceans are adapted to anoxic conditions in OMZs and
47 may have a more direct role in marine nitrogen loss than previously suspected.

## Introduction

In marine oxygen minimum zones (OMZs), the concentrations of dissolved oxygen (DO) can fall to less than 10 nanomoles/L (Bertagnolli and Stewart, 2018). Although they account for only 1% of the ocean by volume, microbial processes in OMZ catalyze 30-50% of marine nitrogen loss (Codispoti, 2010; Lam and Kuypers, 2011). This outsized impact on the marine nitrogen budget is due largely to the anaerobic microbial processes of denitrification and anaerobic ammonia oxidation (anammox) that occur in these low oxygen waters (Kuypers et al., 2005; Lam et al., 2009; Ward et al., 2009).

Denitrification is the stepwise reduction of nitrate ($NO_3^-$) to nitrite ($NO_2^-$), nitric oxide (NO), nitrous oxide ($N_2O$), and di-nitrogen gas ($N_2$) (Zumft, 1997). Each step in the denitrification pathway requires a specific enzyme, and it is common for microbes to possess some, but not all, of the required enzymes for denitrification (Pold et al., 2025; Zhang et al., 2023). While many anaerobic or facultatively anaerobic bacteria can respire nitrate, fewer have the genetic potential to reduce nitrite to a gas (NO, $N_2O$, or $N_2$). This incomplete denitrification can lead to the accumulation of intermediates in anoxic waters and fuel competition between microbes specializing in components of the denitrification pathway (Sun et al., 2024). Low oxygen waters with these microbes can be either a source or sink of $N_2O$, Depending on environmental conditions and interactions among microbial community members.

The discovery that SAR11, one the most abundant lineage of bacteria in the oceans (Morris et al., 2002), have and express genes to reduce nitrate to nitrite greatly expanded their known range beyond the oxygenated ocean and raised their perceived importance in anoxic waters of marine OMZs (Tsementzi et al., 2016; Ruiz-Perez et al., 2021). Evidence that *Pelagibacterales* (SAR11) respire nitrate and produce nitrite in the Eastern Tropical North

71 Pacific (ETNP) OMZ also linked their activities to the dominant nitrogen loss processes in the
72 oceans: denitrification and anammox. However, less is known about the genetic potential and
73 distribution of facultatively anaerobic SAR11 in other OMZs.

74 The Benguela Upwelling System (BUS) is an important and relatively understudied OMZ
75 in the South Atlantic Ocean. Several studies have documented significant production of nitrous
76 oxide within the low-oxygen waters of the BUS (Frame et al., 2014; Arévalo-Martinez et al.,
77 2019; Sabbaghzadeh et al., 2021; Dangl et al., 2025), but the microorganisms responsible for this
78 nitrogen loss, particularly through denitrification, are still largely unknown (Dangl et al., 2025).
79 Sulfur oxidizing γ-proteobacteria from the family *Thioglobaceae* (SUP05) have the potential to
80 respire nitrogen oxides (reviewed in Morris and Speitz, 2022) and often dominate marine
81 microbial communities in the BUS (Lavik et al., 2009). Many other heterotrophic bacteria that
82 are facultatively anaerobic can also respire nitrogen oxides. To identify the potential for bacteria
83 to contribute to denitrification in the BUS, we cultured and sequenced the complete genomes of
84 24 bacteria from low dissolved oxygen waters (24 μM) in the northern BUS (nBUS) OMZ.
85

86 **Results and Discussion**

87 *Diversity of Denitrification Genes in Bacteria from the nBUS*

88 Marine bacteria isolated from the nBUS include diverse members of the
89 *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidia* (**Figure 1**). The isolates have
90 circular chromosomes with genomes that range in size from 1.3 to 4.6 Mbp and have GC
91 contents between 29.6 to 52.5% (**Supplementary Table S1, Supplementary Material**). Most
92 (83%) have the genetic potential to carry out some septs in the denitrification pathway. Only one
93 isolate, *Pseudopelagicola* nBUS_20, has a full set of genes for complete denitrification,

94 suggesting that it has the potential to reduce $NO_3^-$ to $N_2$ (**Figure 1, Supplementary Table S2,**

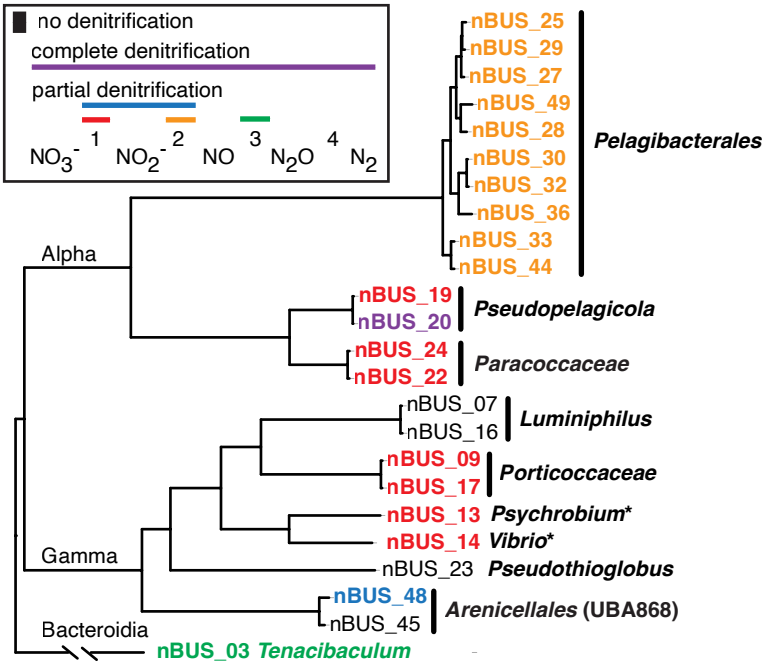
95 **Supplementary Material**). The complete denitrification pathway in nBUS_20 includes both

96 periplasmic *napAB* and respiratory *narGHI* dissimilatory nitrate reductase genes, as well as a

97 *nirS* nitrite reductase, *norC* nitric oxide reductase, and *nosZ* nitrous oxide reductase. The genome

98 of the only *Bacteroidia* isolate, *Tenacibaculum* nBUS_03, codes for a single denitrification gene,

99 NorC nitric oxide reductase.




100

101 **Figure 1**. Genetic potential for denitrification in the genomes of bacteria isolated from the

102 nBUS. Key denitrification genes include those for nitrate reductases (*napAB* and *narGHI*), nitrite

103 reductase (*nirK*), nitric oxide reductase (*norC*), and nitrous oxide reductase (*nosZ*). Asterisks

104 indicate the presence of assimilatory *nasC* and *nirBD* genes. Genes correspond to KEGG

105 orthology numbers are listed under gene_id in Tables S2 and S4: *napAB* (K02567, K02568);

106 *narGHI* (K00370, K00371, K00374); *nirKBD* (K00368, K00362, K00363); *norC* (K02305);

107 *nosZ* (K00376); and *nasC* (K00372).

108 Genes to respire only nitrate and nitrite were identified in one genome and to respire only
109 nitrate were identified in seven genomes. Isolate nBUS_48 is from a previously uncultured
110 family in the order *Arenicellales*, UBA868 (Baltar et al., 2023), which encodes *narGHI* and *nirK*
111 dissimilatory nitrate and nitrite reductase genes. The *Vibrio* nBUS_14 genome encodes *napAB*
112 dissimilatory nitrate reductase genes, as well as key components of assimilatory nitrate and
113 nitrite reduction pathways, including the cytoplasmic *nasC* and *nirBD* genes, respectively.
114 *Psychrobium* nBUS_13, has the cytoplasmic *nasC* and *nirBD* genes. The NirBD nitrite
115 reductases have known roles in fermentative dissimilatory nitrate reduction to ammonium
116 (DNRA) (Pandey et al., 2020), but they are generally linked to assimilatory nitrite reduction
117 (Besson et al., 2022). Other key components of assimilatory nitrate reduction (*nasB*) and
118 fermentative DNRA (*narGHI*) were not identified in these isolates, which suggests that the
119 combination of cytoplasmic systems found in nBUS_13 and nBUS_14 may represent an
120 alternative pathway for nitrate reduction to ammonium.
121 Genes for nitrate respiration were broadly distributed in the nBUS bacterial community.
122 For example, three *Alphaproteobacteria* and four *Gammaproteobacteria* were identified with the
123 genetic potential to respire nitrate (**Figure 1**). The *Alpharoteobacteria* include *Pseudopelagicola*
124 nBUS_19 and two representatives from a previously uncultured genus in the family
125 *Paracoccaceae*, nBUS_22 and nBUS_24. The *Gammaproteobacteria* include two
126 representatives from an undescribed genus in the family *Porticoccaceae* (Stingl et al., 2007),
127 nBUS_09 and nBUS_17, *Psychrobium* nBUS_13, and *Vibrio* nBUS_14.
128

129 *A Conserved NirK-cbb3 Genomic Region in nBUS SAR11*

130 Ten new SAR11 species and subspecies were isolated and sequenced from the nBUS
131 (**Figure 2**). They are members of subclade 1a.III.IV (**Figure 2a, Supplemental Material**),
132 which is a lineage that was recently assigned the genus *Proprepelagibacter* (Freel et al., 2024).
133 Phylogenomic and whole genome average nucleotide identity (ANI) analyses indicate that the
134 nBUS SAR11 represent six new species and four new subspecies, with pairwise ANI values that
135 range from 0.85-0.98 (**Supplemental Table S3, Supplemental Material**). The most striking
136 feature in the nBUS SAR11 genomes is that all ten have a conserved genomic region containing
137 a copper-containing nitrite reductase (*nirK*) and components of cbb3-type cytochrome c oxidases
138 (*cco*) that are not present in the complete genomes of SAR11 cultured from oxygenated waters in
139 the North Pacific, North Atlantic, and Indian oceans (**Figure 2b; Supplemental Table S4,**
140 **Supplementary Material**). Notably, the combined percent GC for *nirK*-cbb3-type cytochrome c
141 oxidase genes in all ten SAR11 is 35.7%, which is higher than the average for the genomes
142 (30.1%). This suggests that the *nirK-cbb3* genomic region in SAR11 is a genomic island that was
143 likely acquired by horizontal gene transfer.

144 Genes for the cbb3-type cytochrome c oxidase are arranged in a canonical operon
145 (Decluzeau et al., 2008) that includes the catalytic subunit (*ccoN*), the cytochrome subunits
146 involved in electron transfer (*ccoO* and *ccoP*), and a small subunit with an unknown function
147 (*ccoQ*). This region also has accessory genes *ccoGHIS* (Koch et al., 2000). The four genes
148 between *nirK* and the *ccoN* code for a protein of unknown function, a pseudoazurin, a Fe(II)/2-
149 oxoglutarate oxygenase, and subunit beta of ribonucleotide reductase (R2 RNR). Of these, only
150 pseudoazurins have known roles contributing to denitrification (Fujita et al., 2012).

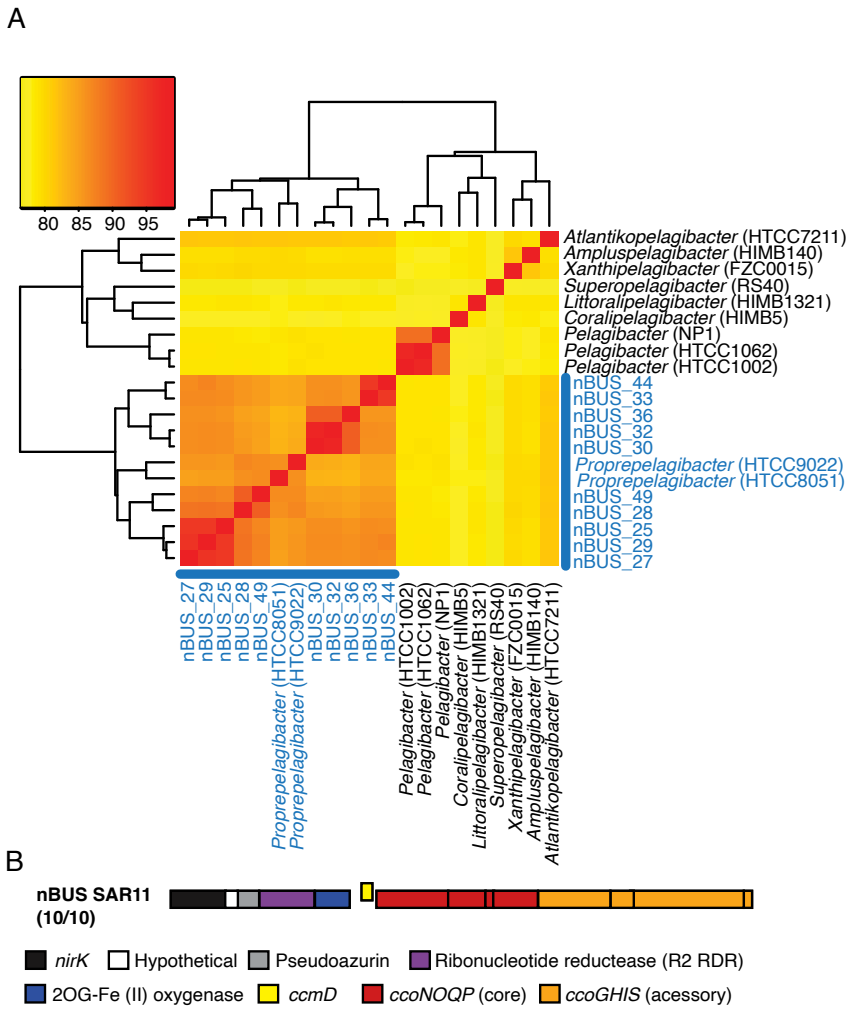



**Figure 2.** Characteristics of SAR11 isolated from the northern Benguela Upwelling System (nBUS). A) Pairwise average nucleotide identity (ANI) analysis of nBUS SAR11 genomes and the genus *Proprepelagibacter* (blue) and other *Pelagibacter* genera (black), and C) genomic region with denitrification and cbb3-typ cytochrome c oxidase genes in nBUS SAR11 genomes. Labels in panel A correspond to genera designations (Freel et al., 2024) and nBUS isolate IDs. IDs are: *nirK*, copper containing nitrite reductase; *ccm*, cytochrome c biogenesis; and *cco*, high affinity cytochrome c oxidase. Genes correspond to KEGG orthology numbers are listed under gene_id in Tables S2 and S4: *nirK* (K00368, K00362, K00363); *ccoNOPQ* (K00404-K00407).

161 None of the nBUS SAR11 genomes code for a nitric oxide reductase (NOR). However,
162 Cco proteins share significant homology with NORs, which has led to considerable debate over
163 the evolutionary origin of cbb3-type cytochrome c oxidases, especially regarding their roles in
164 the evolution of aerobic respiration (Ducluzeau etal., 2008; Gribaldo et al., 2009). This also
165 raises the possibility that some extant cbb3-type cytochrome c oxidases could be bifunctional
166 enzymes that reduce oxygen under oxic conditions and NO to $N_2O$ under anoxic conditions. It
167 has been known for some time that cbb3 heme copper oxidases can reduce NO to $N_2O$ in vivo
168 (Forte et al., 2002). Interestingly, two of the genes between *nirK* and cbb3-type cytochrome c
169 oxidase are related to proteins that bind NO. The Fe(II)/2-oxoglutarate clavamate synthase from
170 *Streptomyces clavuligerus* (Zhang et al., 2002) and the R2 protein of ribonucleotide reductase
171 from *E. coli* (Haskin et al., 1995). The R2 protein in *Escherichia coli* not only binds NO but also
172 produces $N_2O$ in vivo. Regardless, this OMZ adaptation may grant SAR11 the genetic potential
173 to grow under both microaerophilic and anoxic conditions, contributing to nitrogen loss in the
174 nBUS, and potentially other OMZs.
175
176 *Conclusion*
177 Genomic evidence obtained in this study suggests that denitrification potential in the
178 nBUS is distributed among a diverse community of ubiquitous and abundant lineages of marine
179 bacteria, including SAR11 and UBA868. No chemoautotrophic SUP05 with the genetic potential
180 to respire nitrogen oxides were cultured in this study. However, most of the bacteria isolated and
181 sequenced in this study have the genetic potential to contribute to the initial steps in
182 denitrification, either nitrate or nitrite reduction (**Figure 1**). Notably all organisms with genes to
183 respire nitrate or nitrite also have genes for cbb3-type cytochrome c oxidases, indicating the

184 potential to respire oxygen at low concentrations. Relatively few have the genetic potential to
185 contribute to more than one step in denitrification and only one isolate was identified with the
186 genetic potential for complete denitrification (*Pseudopelagicola* nBUS_20). Evidence that
187 SAR11 have genes to reduce nitrite to NO, and possibly NO to $N_2O$ under some conditions,
188 suggests that they could be a critical link in denitrification. Similarly, members of a ubiquitous
189 family of sulfur oxidizing mixotrophs in the order *Arenicellales* (Balter et al., 2023) have the
190 potential to respire nitrate and nitrite. This suggests that genome flexibility is a key to the success
191 of these globally distributed marine bacteria.
192

193 **Materials and Methods**

194 *High-Throughput Cultivation*

195 We used a high-throughput dilution to extinction approach to isolate bacteria from a low
196 dissolved oxygen (24 µmol/kg) sample collected on April 15, 2024 at a depth of 225 m in the
197 BUS (22°59'58.2"S 13°30'E), as previous described (Monaghan et al., 2020). Briefly, 1 ml of
198 seawater was amended with filter-sterilized glycerol to a final concentration of 10% (v/v), flash
199 frozen in liquid nitrogen, and stored at -80 °C. The sample was later thawed on ice and cells were
200 diluted to 5 and 50 cells/ml in 30 kDa filter-sterilized Puget Sound seawater and amended to 100
201 µM thiosulfate. Two mL of the diluted cell suspension was then added to each well of a 96 well
202 Teflon plate and incubated at 13 °C in the dark for four weeks. Wells were screened for growth
203 each week using a Guava easyCyte flow cytometer (Cytek, Fremont, CA). Bacteria in wells that
204 were positive for growth (>$10^5$ cells/ml) were transferred to 1 L acid-washed and autoclaved
205 polycarbonate bottles containing filter-sterilized seawater amended to 100 µm thiosulfate and
206 monitored for growth until they reached between $10^5$ and $10^6$ cells/ml. Cells were then filtered

207 onto 47 mm 0.2 µm isopore membrane filters (MilliporeSigma, Burlington, MA) and stored at -
208 80 °C.
209
210 *High Molecular Weight DNA Extraction*
211 Filters with cells from cultures were placed in sterile 2 mL DNA LoBind tubes
212 (Eppendorf, Hamburg Germany) and cut into smaller pieces with sterile scissors. Sterile TE
213 buffer (200 µL) was added, and filters were frozen at -80 °C for 20 minutes and then thawed at
214 room temperature. High molecular weight DNA was extracted from the filters using a modified
215 Autogen Quick Gene DNA Tissue Kit (Autogen, Holliston, MA), modified by doubling all
216 recommended extraction volumes and eluting DNA in 200 µL of molecular grade water. DNA
217 was purified using 1X v/v DNA magnetic beads (Sergi Lab Supplies, Seattle, WA) with two
218 80% ethanol washes then eluted in 20 µL of molecular grade water. Purified DNA was
219 quantified using a Qubit dsDNA Quantitation High Sensitivity kit (Invitrogen, Waltham, MA).
220
221 *Long-read Genome Sequencing and Assembly*
222 High molecular weight DNA libraries for whole genome sequencing were prepared using
223 the SQK-RAD114 rapid library prep kit (Oxford Nanopore Technologies, Oxford, UK) and
224 sequenced on a Minion equipped with Flongle R10.4.1 flow cells. DNA bases were called using
225 the Dorado basecalling algorithm (dna_r10.4.1_e8.2_400bps_hac@v4.3.0).
226 Genomes were assembled from fastq files with Flye v2.9.1 (Kolmogorov et al., 2019) and
227 polished with Medaka 1.7.2 (github.com/nanoporetech/medaka) using the galaxy web platform
228 (Community, 2024). Single circular contigs were evaluated using DFAST for the initial
229 identification and characterization (dfast.ddbj.nig.ac.jp)(Tanizawa et al., 2018).

230

231 *Genome Annotation and phylogenomic analyses*

232 The final annotation was completed by NCBI using the Prokaryotic Genome Annotation
233 Pipeline v6.8 (Tatusova et al., 2016; Li et al., 2021). Bacterial taxonomy was determined using
234 GTDB-Tk v2.3.2, the de-novo workflow and the v214 reference database (Chaumeil P-A, et al.,
235 2022). All isolates were given the prefix "nBUS" followed by the cultivation number. SAR11
236 species were identified by average nucleotide identity (ANI) with Pan-genome Explorer
237 (Dereeper et al., 2022; Perrin and Rocha, 2021). Phylogenetic trees were constructed with
238 MUSCLE v3.8.31 (Edgar RC. 2004) and RAxML v8.2.11 with model GTRGAMMA
239 (Stamatakis A, 2014) using the ETE3 v3.1.3 phylogenetic analysis pipeline (Huerta-Cepas J, et
240 al., 2016). Whole genome phylogenies were constructed using the bacterial_71 single-copy core
241 gene collection in anvi'o v8 (Eren et al., 2021). Genomes were annotated for comparative
242 analysis of denitrification genes using DRAM (ver. 1.1.1) (Shaffer et al., 2020) with the KEGG
243 (Kanahisa et al., 2023), UniRef90 (Suzek et al., 2015), PFAM (Mistry et al., 2021), dbCAN (Yin
244 et al., 2012), and MEROPS (Rawlings et al., 2018) databases.

245

246 **Supplementary Material**

247 Supplementary material is available at *Genome Biology and Evolution* online.

248

249 **Acknowledgements**

250 We would like to thank the captain and crew of the R/V Marcus G. Langseth and the chief

251 scientist and science party for help with sample collection (MGL24-05).

252

253 **Funding**

254 Field sampling was funded through National Science Foundation awards to OUM and KLC

255 (2113936 and 2113937). Culturing and sequencing work was funded through National Science

256 Foundation awards to RMM (2201310 and 2333925). KB acknowledged funding from the UW

257 Astrobiology Research Rotation program.

258

259 **Data Availability**

260 Genome sequences were deposited in GenBank under Bioproject PRJNA1173757 with accession

261 and sequence read archive identifiers for each genome (**Table S1, Supplemental Material**).

262

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## Author Notes

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