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Phylogenetic similarity of aerobic gram-negative halophilic bacteria from a deep-sea hydrothermal mound and Antarctic habitats

Takuji Okamoto¹, Kantaro Fujioka² and Takeshi Naganuma^{1,2*}

¹*School of Biosphere Sciences, Hiroshima University, Higashi-hiroshima 739-8528*

²*Japan Marine Science and Technology Center, Yokosuka 237-0061*

Abstract: Deep-sea halophilic strains, 4 halomonads and 1 pseudoalteromonad, were isolated from high-temperature hydrothermal fluids of the TAG hydrothermal mound in the Mid-Atlantic Ridge. Two of the TAG halomonads were closely related to Antarctic halomonads based on 16S rDNA sequences (1350 bp). Sub-hydrothermal vents and Antarctic terrains are known to provide high-salinity habitats for halophilic life. The TAG-Antarctic halomonad kinship indicates the wide distribution of halophiles over globally distant habitats, regardless of large differences in temperatures of the habitats. This suggests that microbial eco-physiology in Antarctica (and sub-hydrothermal vent), which has been studied in terms of temperature adaptation, may be complemented by halotolerance and halophilism studies.

key words: 16S rDNA, phylogenetics, hydrothermal vent, Antarctic, *Halomonas*

Introduction

The physical conditions of Antarctica for life are characterized by low temperature and low availability of liquid water for life. Even visible solid water, *i.e.* snow and ice, is not present in certain regions of Antarctica due to 'blowing snow' caused by strong katabatic winds over snow and ice surfaces (Bromwich and Stearns, 1993). An example is the McMurdo Dry Valleys of southern Victoria Land, comprising the largest ice-free expanse (about 4000 km²) on the Antarctic continent. The McMurdo Dry Valleys represents the coldest and driest desert on the globe (Priscu, 1998). Deserts, hot or cold, are often associated with hypersaline conditions, and have been sources for isolation of halophilic (salt-loving) microorganisms (Vreeland and Hochstein, 1993; Oren, 1999).

More than 200 halotolerant and halophilic bacterial strains which have been isolated from various habitats were reported by Herbert and Vreeland (1987), and many species have been added thereafter (Ventosa *et al.*, 1998). Although most of the strains were isolated from rather limited saline to hypersaline habitats, the distribution of halophilic bacteria as a whole is global, from Antarctic sea ice to sea-drifting tar ball and deep-sea sediment. Here we add a new habitat source, hydrothermal vents, for isolation of moderately halophilic bacteria.

The sub-seafloor structure of a hydrothermal vent is highly complex with mosaic heterogeneity of physico-chemical parameters such as temperature and salinity (Alt,

* E-mail address: takn@hiroshima-u.ac.jp

1995). The salinity of the emitted hydrothermal fluid is known to vary on a day-to-week time scale, which is explained by the mixing of low- and high-salinity (brine) fluids (Von Damm *et al.*, 1995). Sub-vent formation of low- and high-salinity fluids is explained by phase separation of super-critical and sub-critical fluids at the critical point (374.1°C and 22.1 MPa for pure H₂O) (Von Damm, 1995). Thus, sub-vent habitats for halophilic life, thermo- or psychrophilic, are possible. This idea is supported by the isolation of autochthonous halophilic residents.

This communication presents preliminary results on the isolation of halophilic bacteria from the TAG hydrothermal mound in the Mid-Atlantic Ridge, and a phylogenetic comparison with halophilic bacteria isolated from Antarctic habitats. Close phylogenetic similarity between some of the TAG and Antarctic *Halomonas* strains is pointed out. Some species of *Halomonas* are known to be able to grow over a wide range of salinity and thus are distributed over a wide range of habitats (Ventosa *et al.*, 1998). Distribution of *Halomonas* and other halophilic bacterial species/strains is discussed in the views of autochthony and allochthony: autochthony for considering TAG-Antarctic habitat similarity, and allochthony for examining entrainment of ubiquitous halophiles.

Materials and methods

Sample collection

High-temperature hydrothermal fluids (194°C and 278°C) were collected at the TAG hydrothermal mound, 3650 m deep, on the Mid-Atlantic Ridge at 26° N, 45° W (Rona *et al.*, 1993; Rona and Von Herzen, 1996) by the manned submersible Shinkai 6500 during the MODE'98 Expedition (Fujioka *et al.*, 1999). The hydrothermal fluids were collected using the ORI rotatory-pump fluid sampler (Tsunogai *et al.*, 1994). Chemical analyses of other TAG fluid samples collected in the same way demonstrated that entrainment of ambient seawater in the fluid samples was only little, possibly less than 10% (Chiba *et al.*, 1999). The possibility of the entrainment of ambient water-derived microorganisms is discussed later.

Bacterial isolation and characterization

Immediately after the fluid collection, each 5 ml of the hydrothermal fluid samples was added to 10 ml of a HAB-BART medium (Heterotrophic Aerobic Bacteria-Bacterial Activity Test; HACH Company, Loveland, Colorado) to which 30% NaCl (w/v) was added. The composition of the medium is proprietary information, however, the material safety data sheet (<http://www.dbi.sk.ca/Droycon/Msds/HAB-MSDS.pdf>) gives the names of the chemical ingredients: glycerol, peptone, tryptone, sodium thiosulfate, magnesium sulfate, and dipotassium phosphate. The final salinity of the culture was >20% NaCl (w/v).

These primary cultures were incubated at room temperature in the dark. After >4 months of incubation, on-board cultures were transferred and further enriched at room temperature in the 1/2 LBG and 1/20 LBG [on a 1/2 basis, bacto-peptone 5.0 g, yeast extract 2.5 g, D-glucose 1.0 g, 5N-NaOH 200 µl per liter of distilled water] liquid medium with different NaCl concentrations from 0 to 30% w/v, and 5 strains of

moderately halophilic bacteria were isolated; 4 strains (C2, C4, C7 and C11) were isolated from the 194°C fluid culture, and 1 strain (F1) was isolated from the 278°C fluid culture. Three cultures with 5 ml of ambient seawater showed no growth at the salinity of >20% NaCl (w/v), which suggests that the halophilic strains were likely derived from the hydrothermal fluids or vicinity. The isolated strains were characterized in terms of gram-staining, salinity-dependent growth, and 16S rDNA sequence. All of the halophilic strains were gram-negative. Their halophilic nature was confirmed by the salinity-dependent growth (See Results and Discussion; Fig. 1).

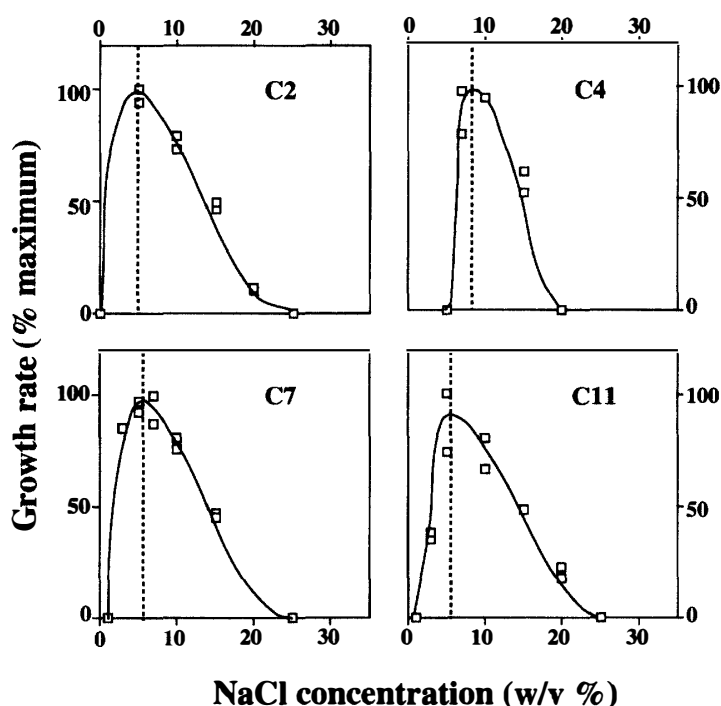


Fig. 1. Salinity-dependent growth of halophilic bacterial strains from the TAG hydrothermal mound on the Mid-Atlantic Ridge. The maximal (100%) doubling times of the strains C2, C4, C7 and C11 were 0.92 hrs, 1.74 hrs, 1.22 hrs, and 1.34 hrs, respectively.

Phylogenetic analysis

Total DNA was extracted from the isolates by DNA Purification Kit (Toyobo Co., Osaka, Japan), and partial 16S rDNA (*ca.* 1502 bp) was amplified by PCR with the primers Eubac27F and Eubac1492R (DeLong, 1992). The 16S rDNA fragments were sequenced by the dideoxynucleotide chain termination method using a model 373A DNA Sequencer (PE Biosystems Co., Foster City, USA). The primers used for sequencing are described by Takami *et al.* (1999).

A phylogram of the selected aerobic gram-negative halophilic bacteria was constructed based on the 16S rDNA sequences (Fig. 2). Partial 16S rDNA sequences, corresponding to the 38–1414 positions of the *Escherichia coli* 16S rRNA gene (Brosius *et al.*, 1978), were aligned using the CLUSTAL W multiple-alignment program (Thompson *et al.*, 1994). Positions with gaps were ignored by using the TOSSGAPS

function of the CLUSTAL W. The phylogram was constructed using the TreeView (Page, 1996) by the neighbor-joining method after 1000 bootstraps.

The 16S rDNA sequences of the TAG strains are registered in the DNA Data Bank of Japan (DDBJ), and the accession numbers are: C2, AB042500; C4, AB042501; C7, AB042502; C11, AB042503; and F1, AB042504. To construct the phylogenetic tree, the 16S rDNA sequences of the gamma-subdivision of the Class Proteobacterial halophiles were collected from the DDBJ, European Molecular Biology Laboratory, GenBank and the Ribosomal Database Project database.

Results and discussion

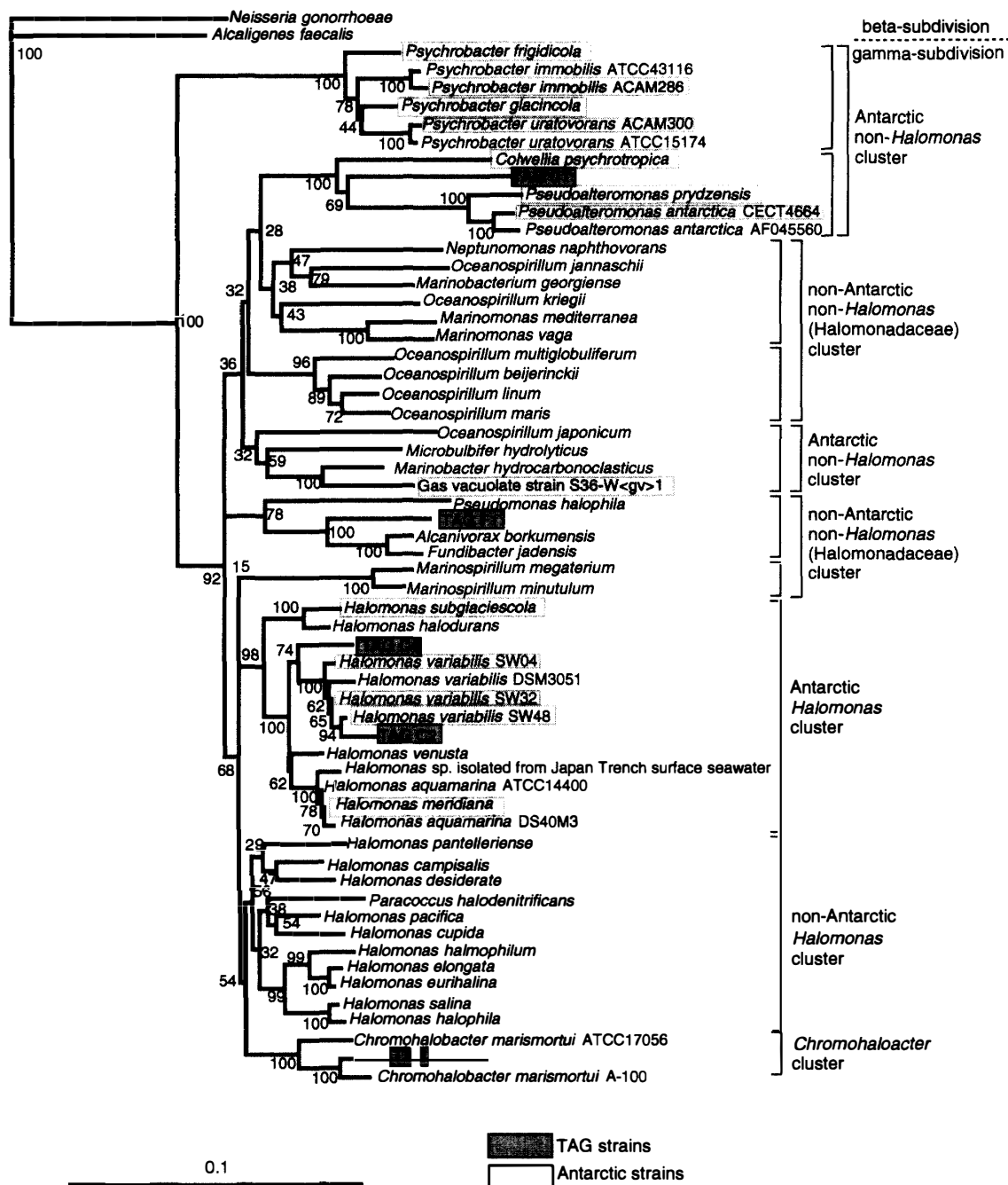
Salinity-dependent growth of the TAG halophiles

Growths of the halophilic strains C2, C4, C7 and C11 were observed within a wide range of NaCl concentrations from 1–7 to 15–25% (w/v) as shown in Fig. 1. The optimum NaCl concentrations of the halophilic strains growths were 6–8% (w/v), which indicates that the isolates are moderate halophiles (Imhoff, 1993). The growth of the C2 strain in the 1/2-strength LBG was seen over a wide range of salinity from 1 to 25% (w/v) NaCl; the optimum NaCl concentration (w/v) of the C2 growth was 6

Fig. 2 (opposite). Phylogenetic tree based on partial 16S rDNA sequences (ca. 1375 bp) of aerobic gram-negative halophilic bacteria including strains from the TAG hydrothermal mound and Antarctic habitats. The sequences of *Neisseria gonorrhoeae* (X07714) and *Alcaligenes faecalis* (AJ277669) belonging to the beta-subdivision of Class Proteobacteria were used as the outgroup. The scale represents the average number of nucleotide substitutions per site. Bootstrap values (%) were calculated from 1,000 trees and are shown at the branch points. The accession numbers of reference organisms used in the phylogenetic analysis are as follows: *Alcanivorax borkumensis* (accession number, Y12579), *Balneatrix alpica* (Y17112), *Chromohalobacter marisumortui* ATCC17056 (X87219), *C. marisumortui* A-100 (X87221), *Colwellia psychrotropica* (U85846), *Fundibacter jadensis* (AJ001150), Gas vacuolate strain S36-W<gv>1 (Gosink and Staley, 1995) (U14584), *Halomonas aquamarina* ATCC14400 (M93352), *H. aquamarina* DS40M3 (AF199439), *H. campisalis* (AF054286), *H. cupida* (L42615), *H. desiderata* (X92417), *H. elongata* (M93355), *H. eurihalina* (X87218), *H. halodurans* (L42619), *H. halmophilum* (M59153), *H. halophila* (M93353), *H. meridiana* (M93356), *H. pacifica* (L42616), *H. pantelleriense* (X93493), *H. salina* (AJ243448), *H. subglaciescola* (M93358), *H. variabilis* DSM3051 (M93357), *H. variabilis* SW04 (U85871), *H. variabilis* SW32 (U85872), *H. variabilis* SW48 (U85873), *H. venusta* (L42618), *Halomonas* sp. isolated from Japan Trench (Maruyama *et al.*, 2000) (AB016049), *Marinobacter hydrocarbonoclasticus* (AB021372), *Marinobacterium georgiense* (U58339), *Marinomonas mediterranea* (AF063027), *Mm. vaga* (X67025), *Marinospirillum megaterium* (AB006770), *Ms. minutulum* (AB006769), *Microbulbifer hydrolyticus* (U58338), *Neptunomonas naphthovorans* (AF053734), *Oceanospirillum beijerinckii* (AB006760), *O. jannaschii* (AB006765), *O. japonicum* (AB006766), *O. kriegii* (AB006767), *O. linum* (M22365), *O. maris* (AB006771), *O. multiglobuliferum* (AB006764), *Paracoccus halodenitrificans* (L04942), *Pseudoalteromonas antarctica* CECT 4664 (X 98336), *Pseudoalteromonas antarctica* (AF045560), *Pseudoalteromonas prydzensis* (U85855), *Pseudomonas halophila* (AB021383), *Psychrobacter frigidicola* (U46143), *Psy. glacincola* (U46145), *Psy. immobilis* ATCC43116 (U39399), *Psy. immobilis* ACAM286 (U46139), *Psy. uratovorans* ATCC15174 (U46141), and *Psy. uratovorans* ACAM300 (U46142).

%. The Antarctic halophile *Halomonas variabilis*, which is of phylogenetic kin to the strain C2 (Fig. 2), also proliferates over a wide range (0 to >15%) of NaCl concentration (Bowman *et al.*, 1997). The C4 strain was capable of halophilic growth over a slightly narrower NaCl range (7 to 15%) than that of the C2 growth with the optimum concentration of 8% NaCl. The F1 strain was not able to grow in liquid media but formed colonies on the 1/2-strength LBG agar plates containing 1 to 25% (w/v) NaCl.

In addition to salinity, the effect of temperature on the growth of the TAG halophiles was examined, based on the capability of colony formation at 4, 37 and 60°C.



The C2, C4 and F1 strains showed the growth at 4°C. Faster growth at 37°C was shown by all of the TAG halophiles, suggesting their psychrotolerant to mesophilic nature rather than psychrophilic nature. None showed growth at 60°C.

Phylogenetic affiliation of the previously known Antarctic halophiles

All of the Antarctic halophiles were convergently affiliated to limited phylogenetic clusters, namely the Antarctic *Halomonas* cluster and the Antarctic non-*Halomonas* clusters (Fig. 2). The Antarctic *Halomonas* cluster consists only of *Halomonas* species, including 4 strains of *Halomonas variabilis*. Species of the genus *Halomonas* are known to be able to grow over a very wide range of salt concentrations, and thus able to distribute over a wide range of habitats. The genus *Halomonas* as a whole is a phylogenetically monophyletic (Dobson and Franzmann, 1996) but a divergent taxon. In contrast, local communities such as TAG and Antarctic halomonads may rather be phylogenetically converged.

The Antarctic non-*Halomonas* clusters consist of the non-*Halomonadaceae* genera, including the deep-sea cold-adapted genus *Colwellia*, recently proposed genera of *Alcanivorax* and *Fundibacter*, *Pseudoalteromonas*, *Microbulbifer*, *Marinobacter* and the cold-adapted genus *Psychrobacter*.

The common feature shared by the Antarctic and other halophilic strains seems to be psychrophilism rather than halophilism. This view is supported by the distribution of the representative psychrophilic genus *Psychrobacter* in deep-sea and Antarctic waters, which is explained by the global circulation of cold deep waters of polar origin (Maruyama *et al.*, 2000).

Phylogenetic affiliation of the TAG halophiles

Some TAG halophiles are affiliated to the Antarctic clusters (Fig. 2). In particular, the C2 strain was deeply placed in the *Halomonas variabilis* subcluster and thus related to *H. variabilis* at the within-species level. A strain of this species has been isolated from the Great Salt Lake, USA. Therefore, *H. variabilis* may be regarded as having global-wide distribution among Antarctica, an inland salt lake and a deep-sea hydrothermal vent. The C4 strain was affiliated to the Antarctic *Halomonas* cluster, too, but to a different subcluster.

Other halophilic TAG strains were affiliated to the Antarctic non-*Halomonas* cluster. The C7 strain was related to the Antarctic, psychrophilic halophiles of the genera *Colwellia* and *Pseudoalteromonas*. Some *Colwellia* species are known as deep-sea halophiles (DeLong *et al.*, 1997). The connection of halo- and psychrophilic nature allows the *Colwellia* species to distribute throughout Antarctic and deep-sea habitats. This may be true for the genus *Psychrobacter*, as the *Psychrobacter* species are distributed widely in cold deep-sea and Antarctic waters. However, 16S rDNA-based relatedness may not necessarily reflect physiological similarity, and thus the C7 strain may not be psychrophilic.

The exception for the TAG-Antarctic halophilic connection is the strain F1 and C11. However, the F1 strain is related to recently reported halophiles *Alcanivorax borkumensis* (Yakimov *et al.*, 1998) and *Fundibacter jadensis* (Bruns and Berthe-Corti, 1999) isolated from the North Sea, and the strain F1 is psychrophilic halophile. Again,

this is a psychrophilic halophile that is distributed in deep-sea and polar regions, which is explained by the global circulation of cold deep waters of polar origin (Maruyama *et al.*, 2000).

The C11 strain is closely related to strains of *Chromohalobacter marismortui* isolated from hot brine habitats in the Mediterranean and Dead Sea (Ventosa *et al.*, 1989). The genus *Chromohalobacter* is included in the family *Halomonadaceae* (Ventosa *et al.*, 1998), however, no Antarctic species have been reported.

Occurrence of Antarctic-type halophiles at the TAG site

A hypothesis presented in this communication is the occurrence of autochthonous halophiles in the sub-seafloor hydrothermal vent structure, or the sub-vent biosphere. This hypothesis was tested by isolating moderately halophilic strains from the TAG hydrothermal mounds. However, this hypothesis is countered with the view that psychrophilic halophiles are distributed widely in deep-sea and Antarctic waters. Then, the occurrence of halophiles in the TAG hydrothermal fluids may be partly due to the entrainment of common deep-sea psychrophilic halophiles. The TAG halophiles may not necessarily be autochthonous residents in the sub-vent biosphere. This may be true for the presumed Mediterranean-type C11 strain, although it is not clear that the C11 strain is truly a migrant from the Mediterranean.

The discussion may be furnished with the fact that five TAG halophiles capable of growth at 20% NaCl were isolated from total 10 ml ($5\text{ ml} \times 2$) of hydrothermal fluids containing probably <10% ambient seawater; and that no such halophiles were isolated from total 15 ml ($5\text{ ml} \times 3$) of ambient seawater (unpublished data). This is not conclusive evidence but supports the idea that some of the TAG halophiles, if not all, are autochthonous sub-vent residents.

The sub-seafloor structure of a hydrothermal vent is a highly complicated system with mosaic heterogeneity of physico-chemical parameters such as temperature and salinity. This allows the presence of low/high-temperature and low/high-salinity habitats. Some allochthonous halophiles may have resided therein and become autochthonous residents. The TAG halophiles might be regarded as candidate autochthons. Or, they may be, again, ubiquitous and temporarily present at the TAG site at the time of sample collection.

Future scope: allochthonous and autochthonous

The discussion above is only preliminary, based on a small amount of data. The question to be answered is “allochthonous *or* autochthonous?”. To answer this question, an extensive survey of halophilic bacteria occurring in diverse habitats is needed. A tentative answer might be “allochthonous *and* autochthonous”. As a hypothetical remark, the similar phylogenetic affiliation of the TAG and Antarctic halophiles reflects a mixture of ubiquitous deep-sea psychrophilic halophiles (allochthons) and possible permanent residents in the stable brine habitats (autochthons). Isolation and characterization of halophiles such as the *Halomonas* species related to the C2 and C4 strains will facilitate dissecting the allo-/autochthonous view of halophilic microflora.

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