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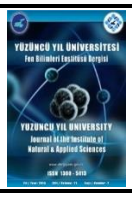

Araştırma Makalesi

Prokaryotic Diversity of a Hypersaline Spring Water in Pülümür (Tunceli)

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Abstract: The microbial diversity of the solar salterns and salt lakes has been extensively studied, but there are only a few reports on microbiota of hypersaline spring waters. In this study, we focused on the archaeal and bacterial groups inhabiting a hypersaline spring water in Pülümür. In addition to culture-dependent studies, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of 16S rRNA genes and fluorescence *in situ* hybridization (FISH) analyzes were performed. The total cell count performed using 4',6-diamidino-2-phenylindole (DAPI) stain revealed ~10^5 cells mL^{-1} in spring water. The FISH analysis showed that bacterial cells accounted for ~72% of the total microbial community, while archaeal cells made up a small portion of the community. Isolates were phylogenetically related with the genera *Rhodovibrio*, *Spiribacter*, *Salinivibrio*, *Halomonas*, *Idiomarina*, *Marinobacter*, *Natrinema* and *Halohasta*. Phylotypes of *Spiribacter* and phylotypes distantly associated with *Owenweeksia* were obtained from DGGE analysis.

Pülümür'deki (Tunceli) bir Hipersalin Kaynak Suyunun Prokaryotik Çeşitliliği

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Öz: Tuz göllerinin ve solar tuzlaların mikrobiyal çeşitliliği kapsamlı bir şekilde çalışılmıştır, ancak hipersalin kaynak sularının mikrobiyotaları hakkında sadece birkaç rapor vardır. Bu çalışmada, Pülümür'deki bir hipersalin kaynak suyunda yaşayan arkeal ve bakteriyal gruplara odaklandık. Kültür-bağımlı çalışmalara ek olarak, 16S rRNA genlerinin PCR ile amplifiye edilmiş fragmentlerinin denatüre edici gradiyent jel elektroforezi (DGGE) ve floresan in situ hibridizasyon (FISH) analizi gerçekleştirildi. DAPI boyası kullanılarak gerçekleştirilen toplam hücre sayımında kaynak suyunda ~10^5 hücre/mL saptandı. FISH analizi, bakteri hücrelerinin toplam mikrobiyal topluluğun ~%72'sini oluşturduğunu, arkeal hücrelerin ise topluluğun küçük bir bölümünü oluşturduğunu göstermiştir. İzolatlar, *Rhodovibrio*, *Spiribacter*, *Salinivibrio*, *Halomonas*, *Idiomarina*, *Marinobacter*, *Natrinema* ve *Halohasta* cinsleriyle filogenetik olarak ilişkiliydi. *Spiribacter* filotipleri ve *Owenweeksia* ile uzaktan ilişkili filotipler DGGE analizinden elde edilmiştir.

1. Introduction

Natural and artificial hypersaline environments are widespread in Turkey. Tuz Lake, the second largest lake in Turkey, is one of the most prominent representatives of these environments. Another representative, solar salterns, are artificially interconnected pool systems to evaporate saline water and produce salt. NaCl precipitates as halite crystals in ponds and is then harvested. Çamaltı and Ayvalık, located on the shore of Aegean Sea, are the major solar salterns consisting of ponds fed with sea water. In the central and eastern part of Anatolia, there are also inland solar salterns fed by the spring water derived from rock salt. During the geological periods, rock salt deposits formed as a result of evaporation of the sea or closed inner basins. As underground water passes through the rock salt layer, it melts some of the salt and emerges to the earth. There are several rock salt mines and hypersaline springs on the salt deposits starting from Çankırı extending to Yozgat, Sivas, Erzincan, Erzurum, Kağızman, and also connected to Iran via Tuzluca (Iğdır) (Güner et al., 2000; Kılıç & Kılıç, 2005).

Halophiles are organisms that need salt for growth, and are grouped into "extreme halophiles" (2.5–5.2 M NaCl), "moderate halophiles" (0.5–2.5 M NaCl) and "slight halophiles" (0.2–0.5 M NaCl) according to their optimum salt requirements. There are also microorganisms that develop optimally at less than 0.2 M NaCl but can tolerate high salt concentrations, and are defined as "halotolerant" (Ventosa et al., 2012). The extremely halophilic archaeal family *Halobacteriaceae* (*Euryarchaeota*), the anaerobic bacterial order *Halanaerobiales* (*Firmicutes*) and the bacterial family *Halomonadaceae* (*Proteobacteria*) are almost entirely composed of halophiles. The representatives of the extremely halophilic bacteria *Salinibacter ruber* (*Bacteroidetes*) and the unicellular green algae *Dunaliella salina* (*Chlorophyta*) have also been frequently detected in hypersaline environments. There are two major mechanisms that halotolerant and halophilic microorganisms use to cope with high osmolarity in their environment. The first strategy, called "salt-in", is based on the accumulation of salts such as KCl into the cell. Cells using this strategy must have intracellular enzyme systems and mechanisms adapted to high salt concentration. The "salt-in" strategy is mainly used by members of *Halobacteriaceae*, *Halanaerobiales* and *Salinibacter ruber*. Microorganisms using the "salt-out" strategy achieve osmotic balance by synthesizing low molecular weight organic solutes or taking it from the surrounding environment. Most of the halophilic and halotolerant bacteria, halophilic methanogenic archaea, eukaryotic algae and fungi use organic compatible solutes to maintain osmotic equilibrium (Oren, 2008, 2010).

There are several reports on the isolation, identification and characterization of halophilic microorganisms from different hypersaline environments in Turkey, such as Tuz Lake, Çankaya salt mine and Çamaltı salterns (Birbir et al., 2007; Yaşa et al., 2008; Mutlu et al., 2008; Yıldız et al., 2012). However, few studies have focused on microbial communities of hypersaline water bodies. In this research, the archaeal and bacterial diversity in hypersaline spring water feeding an inland small solar saltern found in Pülümür district of Tunceli had been investigated.

2. Materials and Methods

2.1. Sampling

Sampling was done in June 2013 from spring water feeding ponds at an altitude of 1750 m (39°30'25.5"N, 39°52'42.6"E). Total salinity of the sample was measured using a hand refractometer (Eclipse). Experimental processes were carried out in a short time after sampling.

2.2. DAPI counts and FISH analysis

The total cell count was performed with DAPI staining, and Cy3-tagged ARC915 and EUB338 oligonucleotide probes were used in the hybridization protocol, allowing us to capture archaeal cells and most bacterial cells (respectively).

For the fixation of the cells (1mL sample), 37% formaldehyde was used with a final concentration of 7%. Sample left at 4°C for 16 h were then diluted with 10 mL of 1X PBS and passed through 0.2 µm pore size filter (Millipore, GTTP). The filter was left to dry at room temperature. Two filters were prepared and analyzed.

A FISH protocol optimized by Antón et al. (1999) for hypersaline aquatic samples was used in the hybridization step. Filters were cut into sections for treatment with probes. First, hybridization with probe (50 ng μL^{-1}) for 2 h, then staining with DAPI (1 $\mu g\ mL^{-1}$) for 5 min processes were applied to the filters.

Cells were observed and counted in at least 25 different microscopic fields on each filter piece by fluorescence microscopy (Leica DM6000 M). Applying probe hybridization and DAPI staining to the same filter allows simultaneous observation and counting of microbial and target cells. Probe-DAPI staining images belonging to the same field of view were checked against each other to reduce false positive hybridization signals. The nonsense probe NON-338 was used as a negative control to test non-specific hybridization.

The parameters taken into account when calculating the number of cells mL^{-1} were as follows: the volume of the filtered sample, the area of the working surface on the filter, average number of cells per microscopic area and the area of the microscopic view.

2.3. Nucleic acid extraction

The Millipore filter (0.22 μm pore size, GTTP) was used for the filtration of the water sample and the extraction protocol described by Mutlu et al. (2008) was carried out. After the filtration process, the filter was cut into small pieces and transferred into eppendorf tubes (2 mL). 600 μL of extraction buffer (100 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added into the tubes. Lysozyme (3 mg mL^{-1}), proteinase K (150 mg mL^{-1}) and 10% sodium dodecyl sulfate were added in order to lyse the cells. The tubes were incubated in a shaker at 37°C. NaCl (5 M) and CTAB (10% CTAB, 0.7 M NaCl) solutions were added. Heat-shock was applied to the tubes using liquid nitrogen and a water bath at 65°C. Phenol-chloroform-isoamyl alcohol (25:24:1) was used in the purification step. After the nucleic acids were precipitated with ethanol, the ethanol was carefully removed and the pellet was resuspended in Milli-Q water after drying in air for a short time.

2.4. DGGE analysis

Polymerase chain reaction (PCR) primer sets used to amplify 16S rRNA genes from total DNA were given in Table 1. Reverse primer with GC-clamp used in PCR. Ingeny system were used for DGGE analysis. Archaeal and bacterial DGGE-PCR products were loaded on DGGE gel containing 40% to 70% denaturing agents (urea and formamide). Electrophoretic separation was carried out at 80 V, 60°C for 18 h. The DNA fragments in the gel were observed by ethidium bromide staining. The bands were excised and resuspended in sterile Milli-Q water. The tubes were kept overnight at 4°C. Re-amplification was carried out with the same primer sets without GC-clamp.

2.5. Cultivation studies

Cultivation studies were carried out using 18% MGM and 23% MGM (Modified Growth Medium) (Dyall-Smith, 2009). Primer sets given in Table 1 were used to amplify the 16S rRNA genes of the isolates. The PCR products were digested with *Hinf*I restriction enzyme to analyze with ARDRA (Amplified rDNA Restriction Analysis) and digested products were visualized by agarose gel electrophoresis. Representatives were selected and sequenced based on ARDRA profiles from 16S rRNA genes (Beckman CEQ 8000 DNA sequencer). The ARDRA analysis and sequencing were done as described by Çınar & Mutlu (2018). The sequences were aligned with MUSCLE (Edgar, 2004) and the phylogenetic tree was created using RaxML program with maximum likelihood algorithm (Stamatakis, 2014).

Sequence data submitted in GenBank database under accession numbers KJ161482-KJ161496 and KY099612-KY099613.

Table 1. Primer sets and conditions used in PCR reactions

PCR conditions	Primers	Primer Sequences and References
For DGGE-PCR		
94°C 5 min, 65°C 1 min, 72°C 3 min (1 cycle)	907r-GC (Universal)	5'-GCclamp-CCGTCAATTCCTTTRAGTTT-3' (Muyzer et al., 1993)
94°C 1 min, 65°C (decreases 1°C per cycle) 1 min, 72°C 3 min (9 touchdown cycles)	344f (Archaea)	5'-ACGGGGCGCAGCAGGCGCGA-3' (Muyzer et al., 1993)
94°C for 1 min, 55°C 1 min, 72°C 3 min (20 cycles)	341f (Bacteria)	5'-CCTACGGGAGGCAGCAG-3' (Muyzer et al., 1993)
72°C, 10 min (1 cycle)		
For 16S rRNA gene amplification of the isolates		
94°C 3 min (1 cycle)	1492r (Universal)	5'-GGTTACCTTGTTACGACTT-3' (Lane et al., 1985)
94°C 15 s, 55°C 30 s, 72°C 2 min (30 cycles)	21f (Archaea)	5'-TTCCGGTTGATCCTGCCGGA-3' (DeLong, 1992)
72°C, 10 min (1 cycle)	27f (Bacteria)	5'-AGAGTTTGATCATGGCTCAG-3' (Lane et al., 1985)

3. Results

This study provides information on prokaryotic groups that are distributed in a hypersaline spring water found in Pülümür. Approaches targeting the 16S rRNA gene have been used to detect and identify archaeal and bacterial groups.

Spring water is transferred to artificial ponds for salt production and an image of the solar saltern was given in Figure 1. Total salinity of the spring sample was measured as 18% and pH as 7.38. The DAPI count revealed that the sample contained $3.3x10^5 \pm 9.0x10^4$ cells mL^{-1} (mean±SD). In FISH analysis, the counting of the archaeal and bacterial cells was performed using domain-specific probes targeting 16S rRNA. No signal detected with NON-338 probe. Bacterial cells were found to be dominant in the microbial community (Figure 2A and 2B). Bacterial and archaeal cells accounted for 72.6% and 0.8% of total microbial cells, respectively. Eukaryotic-like cells were also observed in microscopic analysis.

Figure 1. An image of the solar saltern in Pülümür.

Images of DGGE gels were shown in Figure 2C. The archaeal bands in DGGE gel appear to be quite faint. The FISH analysis revealed that the proportion of archaeal cells in the community was less than 1%. The low amount of DNA belonging to this group in the total DNA extract probably resulted in poor PCR products. Sequencing of these weak archaeal bands failed. Bands with clean sequencing data are marked on the bacterial gel (Figure 2C).

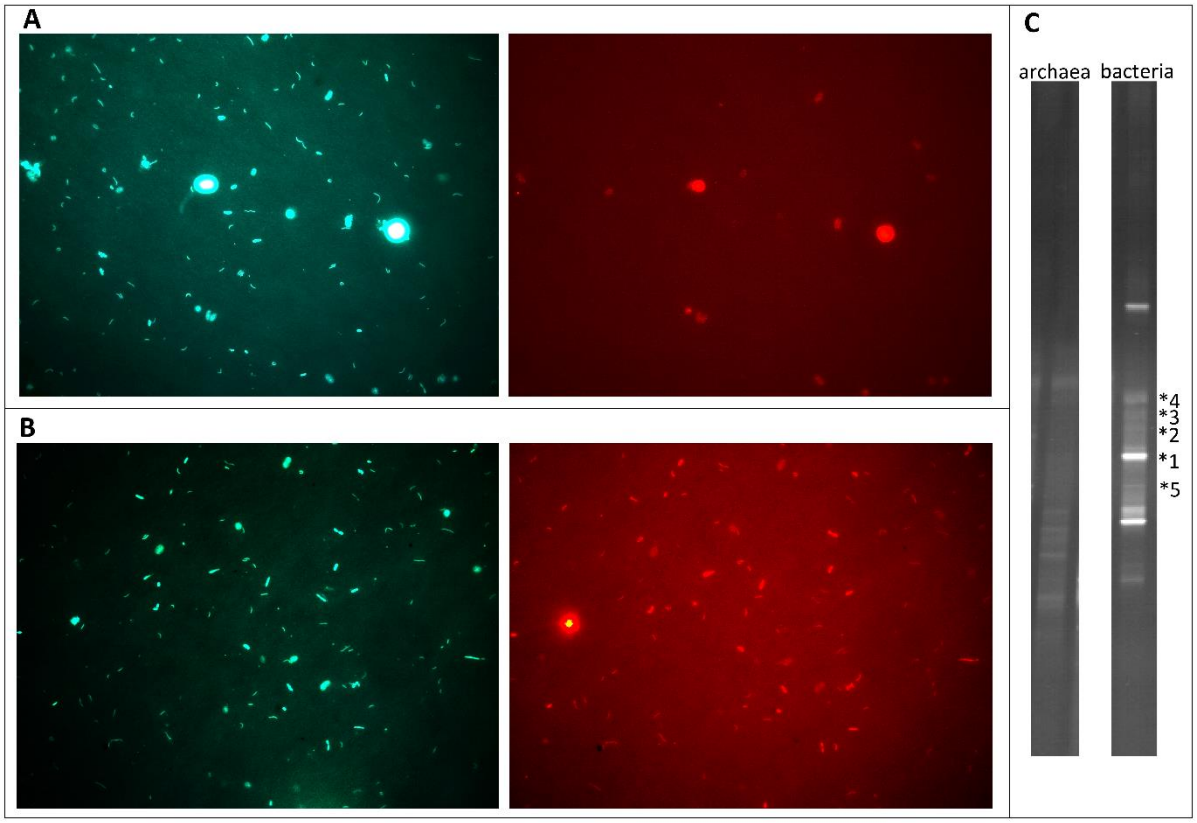

Figure 2. Microscopic images of Pülümür sample. Microbial cells observed by DAPI staining were shown on the left, archaeal cells that hybridized with ARC915 probe (A) and bacterial cells that hybridized with EUB338 probe (B) were shown on the right. DGGE gel images belonging to *Archaea* and *Bacteria* (C). Sequenced bands marked by asterisk (*).

In cultivation studies, 16S rRNA gene PCR products of the isolates analyzed by ARDRA gave 2 different patterns for Archaea and 11 different for Bacteria, and 13 representative strains were selected for further analysis. The isolates were found to be phylogenetically related to *Natrinema*, *Halohasta*, *Rhodovibrio*, *Spiribacter*, *Salinivibrio*, *Halomonas*, *Idiomarina* and *Marinobacter* genera (Figure 3).

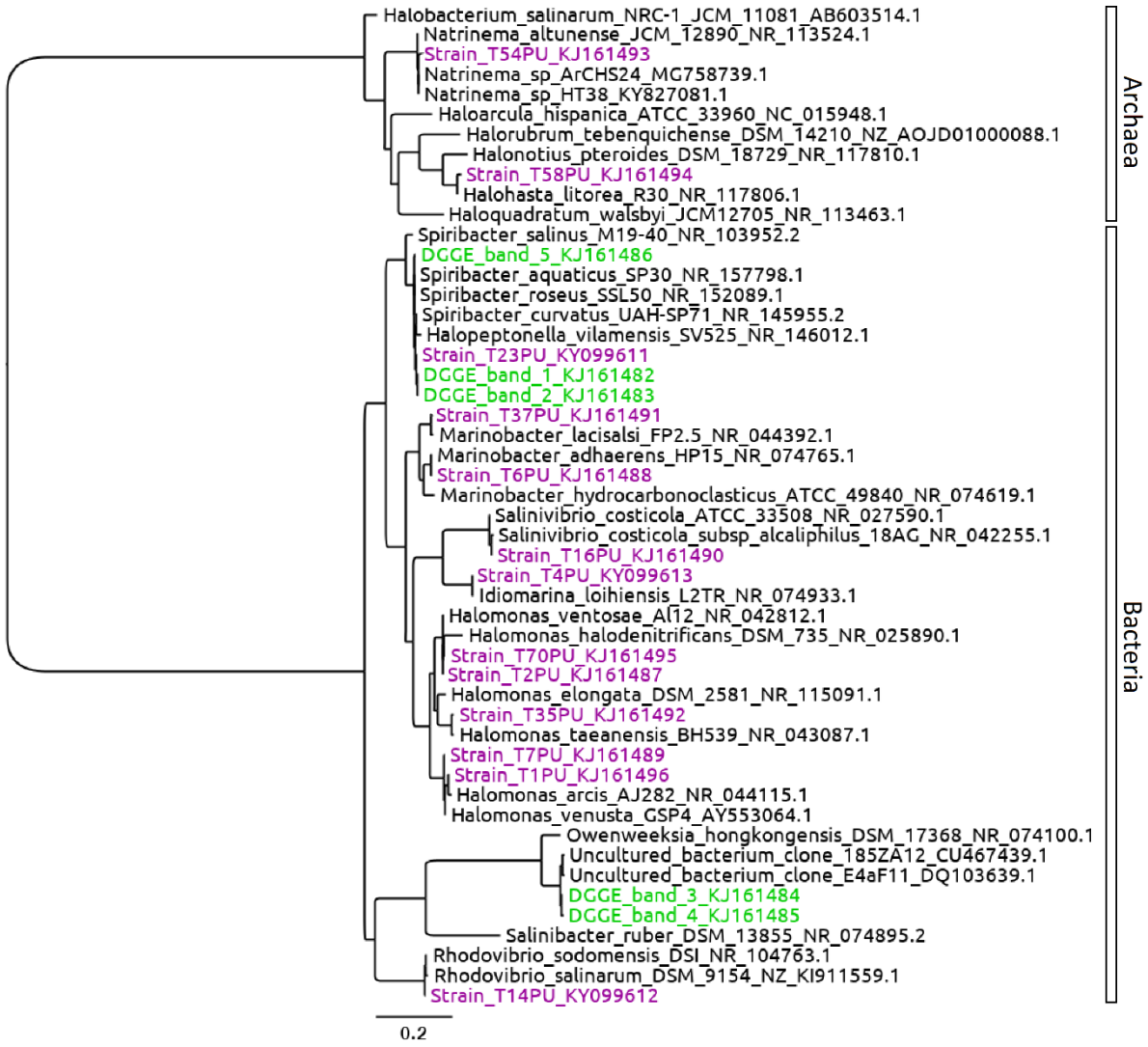

Figure 3. Phylogenetic tree constructed from 16S rRNA genes of the isolates and DGGE bands obtained in this study. Scale bar corresponds to 0.2 changes per nucleotide.

4. Discussion and Conclusion

The number of the microbial cells in different hypersaline spring bodies analyzed in our previous studies was found to be around 10^2-10^3 cells mL^{-1} (Çınar & Mutlu, 2016). Maturrano et al. (2006) reported that microbial cells in spring water feeding an inland solar saltern in Peru were around 100 cells mL^{-1}. Microscopic examination revealed that the cell density in the Pülümür sample was higher than those obtained in previous studies.

The DGGE analysis revealed the existence of novel groups that we could not obtain in the cultivation study. The bands 3 and 4 were found to have a distant similarity (94%) with *Owenweeksia hongkongensis* strain DSM 17368. The sequences of T23PU strain and DGGE bands 1, 2, 5 obtained in this study showed a high similarity (99%) to that of *Spiribacter aquaticus* strain SP30 isolated from a solar saltern (Spain) (León et al., 2017). In previous studies, *Halomonas* and *Salinibacter*-related phylotypes were obtained from Tuz Lake and Çamaltı saltern (İzmir) by DGGE analysis (Mutlu & Güven, 2015; Mutlu et al., 2008). Strains of *Halomonas* were obtained from Pülümür sample, but *Salinibacter*-related phylotypes were not detected in either DGGE analysis or isolation studies. In addition, *Salinibacter* strains could not be obtained from the different hypersaline spring waters that feed the crystallization ponds of the salterns in Erzincan and Sivas (Çınar & Mutlu, 2016).

In cultivation study, we also obtained bacterial strains belonging to the genera *Rhodovibrio*, *Salinivibrio*, *Halomonas*, *Idiomarina* and *Marinobacter*. The majority of the bacterial strains belonged

to the genus *Halomonas*. Strains belonging to all of the specified genera were also obtained in our previous studies from the spring water feeding Tuzlagözü saltern (Sivas, 19% salinity) of which salinity is close to Pülümür spring. In addition, representatives of these bacterial genera were obtained from many different inland and marine solar salterns in Turkey (Mutlu & Güven, 2015; Çınar & Mutlu, 2016 and 2018; Özdemir et al., 2018).

One of the archaeal strains showed 99% sequence similarity with *Halohasta litorea* strain R30 isolated from a brine sample in China and the other 99% with *Natrinema* sp. HT38 isolated from the salt sample of Hamo saltern (Sivas) (Mou et al., 2012; Çınar & Mutlu 2018). We previously isolated halophilic archaeal strains from the hypersaline spring waters in Sivas and Erzincan, including representatives of *Haloarcula* and *Halorubrum* genera (Çınar & Mutlu, 2016). Strains of *Haloarcula*, *Halorubrum*, *Haloferax* and *Halobacterium* were readily recovered from many different hypersaline environments in Turkey (Birbir et al., 2007; Özcan et al., 2007; Mutlu et al., 2008; Yaşa et al., 2008; Yıldız et al., 2012), however could not be obtained from hypersaline spring in Pülümür. Strains of *Natrinema* previously isolated by Özcan et al. (2007) from different hypersaline environments in Turkey, however the strain associated with *Halohasta* genus was obtained for the first time in this study.

Here, we report the prokaryotic groups detected in a hypersaline spring sample (Pülümür). Representatives of *Halobacteriales*, *Alphaproteobacteria* and *Gammaproteobacteria* taxa were isolated and identified. Additionally, phylotypes belonging to *Bacteroidetes* phylum were recovered by DGGE analysis. The presence of microbial genera such as *Halohasta* and *Spiribacter*, which we have not encountered previously in other hypersaline water bodies in Anatolia, was detected, and their representatives were isolated.

Conflicts of interest

The authors declare no conflict of interest.

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