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Exploring the diversity of extremely halophilic archaea in food-grade salts

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ABSTRACT

Salting is one of the oldest means of food preservation: adding salt decreases water activity and inhibits microbial development. However, salt is also a source of living bacteria and archaea. The occurrence and diversity of viable archaea in this extreme environment were assessed in 26 food-grade salts from worldwide origin by cultivation on four culture media. Additionally, metagenomic analysis of 16S rRNA gene was performed on nine salts. Viable archaea were observed in 14 salts and colony counts reached more than 10^5 CFU per gram in three salts. All archaeal isolates identified by 16S rRNA gene sequencing belonged to the *Halobacteriaceae* family and were related to 17 distinct genera among which *Haloarcula*, *Halobacterium* and *Halorubrum* were the most represented. High-throughput sequencing generated extremely different profiles for each salt. Four of them contained a single major genus (*Halorubrum*, *Halonotius* or *Haloarcula*) while the others had three or more genera of similar occurrence. The number of distinct genera per salt ranged from 21 to 27. *Halorubrum* had a significant contribution to the archaeal diversity in seven salts; this correlates with its frequent occurrence in crystallization ponds. On the contrary, *Haloquadratum walsbyi*, the halophilic archaea most commonly found in solar salterns, was a minor actor of the food-grade salt diversity. Our results indicate that the occurrence and diversity of viable halophilic archaea in salt can be important, while their fate in the gastrointestinal tract after ingestion remains largely unknown.

1. Introduction

Salt (sodium chloride, NaCl) is an ingredient extensively used in food preparations. Its history stretches back to antiquity, probably coinciding with the discovery of agriculture and the early means of food preservation (Desrosier, 1970). The use of salt in the food and drink industry has three main roles. First, it gives a specific flavor to the product, enhances and modifies the flavor of other ingredients and reduces the sensation of bitterness. Second, it helps the handling and processing of many products: it makes gluten more stable and less extensible in bread; it regulates the activity of starter culture microorganisms in cheese, increases water-holding capacity and improves the tenderness of meat. Third, and most importantly, salt acts as a food preservative by reducing water activity (Hutton, 2002).

Large-scale processes of salt production include rock salt mining, solution mining, solar evaporation and evaporation with heating devices. At small scale, local extraction of salt is a common practice in rural areas where salt sediments are easily accessed (shallow salt lakes or saline soil crusts). Worldwide production is led by China (24%), Europe (21%) and USA (17%) (The European Salt Producers' Association, 2004). Salt applications for food and feed represent 3% of the European consumption whereas industrial or chemical applications and road de-icing account for 66% and 29%, respectively (The European Salt Producers' Association, 2004).

Solar salterns and aquatic environments with salinities at or near saturation have been widely studied as they maintain high microbial densities (10^7–10^8 cells ml^{-1}) due to the lack of predation and high nutrient concentration (Oren, 2002). These ecosystems have revealed the joint presence of bacteria and archaea, the latter largely dominated by members of the *Halobacteriaceae* family (Benlloch et al., 2002; Oren, 2002). Currently (as of April 2014), 40 genera and 158 species make up this family whose type strains have been isolated from different environmental sources: water and sediment from hypersaline lakes (39%), water from solar salterns (22%), soils and rocks (16%), food (15%) and other sources (8%).

Metagenomic assessments of archaeal diversity in solar salterns from different regions of the globe have often reported the presence of the *Halorubrum* and *Haloquadratum* genera (Burns et al., 2004; Oh et al., 2010; Pašić et al., 2005; Zafrilla et al., 2010), while culture-based methods have identified *Haloarcula*, *Halobacterium*, *Halorubrum* and *Haloferax* as main genera (Benlloch et al., 2001; Oren, 2002; Burns et al., 2004; Birbir et al., 2007; Bidle et al., 2005). This apparent contradiction might be explained by the difficulty to cultivate *Haloquadratum walsbyi*, as opposed to the "rapid" colony formation of *Haloarcula*, *Halobacterium* and *Haloferax* species (Birbir et al., 2007; Burns et al., 2004; Pašić et al., 2005; Rodríguez-Valera et al., 1998).

Salted and fermented food products often contain halophilic archaea. Denaturing gradient gel electrophoresis revealed the presence

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of *Halorubrum* and *Halosarcina* species in brines during fermentation of table olives (Abriouel et al., 2011). Histamine-degrading archaea belonging to genera *Halobacterium* and *Natrinema* were reported in salted-fermented fishery products and *Haloarcula marismortui* was isolated from salted anchovies (Moschetti et al., 2006; Tapingkae et al., 2010). Kimchi, a Korean fermented food, was shown to maintain archaeal population of the *Natronococcus*, *Natrialba*, *Halosimplex*, *Halobiforma* and *Halococcus* genera (Chang et al., 2008). This dish is prepared with jeotgal, a salted-fermented seafood ingredient from which five new species of the *Halobacteriaceae* family were isolated: *Natronococcus jeotgali*, *Halakalicoccus jeotgali*, *Haloterrigena jeotgali*, *Haladaptatus cibarius* and *Halorubrum cibi* (Roh and Bae, 2009; Roh et al., 2007a, 2007b, 2009, 2010).

The salt itself has proven to contain viable microbial cells. In fact, the first food product from which a member of the *Halobacteriaceae* family was isolated, was a food-grade salt from Trapani, Sicily (Petter, 1931). *Halorubrum trapanicum* was originally known as *Halobacterium trapanicum* and was renamed in the course of a rearrangement of the taxonomy of the *Halobacteriaceae* (McGenity and Grant, 1995).

To our knowledge, no studies have investigated the occurrence and diversity of archaea in food-grade salts. Therefore, this study aims to assess the archaeal diversity in 26 food-grade salts from worldwide origin: Europe, Asia, North and South America, Africa and Oceania, by both culture-dependent and independent approaches.

2. Materials and methods

2.1. Food-grade salt samples

A selection of 26 food-grade salts was analyzed in this study (Table 1). Their geographical origins cover many regions of the globe (Fig. 1). Visual aspect reveals an important diversity of color and particle size (Supplementary Fig. S1). Salts were purchased directly from the producer, from local markets or from Belgian supermarkets and organic food e-markets (Supplementary Table S1). They were stored at room temperature in a dark and dry place.

2.2. Culture media

Four media developed to promote archaeal growth were used: MGM, Hv-YPC, DBCM2 and CDM with pyruvate. Their composition and preparation are described below.

2.2.1. Modified growth medium (MGM)

Solid MGM at a salt concentration of 23% was modified from Dyall-Smith (2009). A stock solution of salt water (SW) 30% was prepared as previously described (Porter et al., 2005). 767 ml SW 30% was mixed with 200 ml ultrapure water, 0.5% peptone (Oxoid Ltd., Basingstoke, Hampshire, UK), 0.1% yeast extract (Oxoid Ltd.) and 1 ml trace element solution SL10 (Widdel et al., 1983). After complete dissolution, the pH was adjusted dropwise to 7.5 with Tris base 1 M. The solution was topped up to 1 l with ultrapure water and volumes of 250 ml were dispensed in 500 ml bottles in which 1.5% unwashed agar (MP Biomedicals, Illkirch, France) were added. The bottles were placed in a water bath at 95 °C until complete dissolution of agar. The medium was autoclaved and poured hot into petri dishes.

2.2.2. Hv-YPC

Solid medium Hv-YPC preparation was modified from Dyall-Smith (2009). YPC 10× was prepared by dissolving 5.0% yeast extract (Oxoid Ltd.), 1.0% peptone (Oxoid Ltd.), 1.0% casamino acids (Difco Laboratories, Le Pont de Claix, France) and 17.6 ml KOH 1 M in ultrapure water to a final volume of 1 l. 100 ml ultrapure water, 200 ml SW 30%, 1.5% unwashed agar (MP Biomedicals) and 30 ml YPC 10× were dispensed in 500 ml bottles. The bottles were placed in a water bath at 95 °C until complete dissolution of agar and the medium was autoclaved. After cooling to about 80 °C, 2 ml of filter-sterilized $CaCl_2$ 0.5 M was sterilely added to each bottle. The medium was slowly shaken to avoid bubbles and poured into petri dishes.

2.2.3. Chemically defined medium (CDM) with pyruvate

The preparation of solid CDM medium with pyruvate was modified from Kauri et al. (1990). It was prepared by dissolving 12.5% NaCl, 5.0% $MgCl_2 \cdot 6H_2O$, 0.5% K_2SO_4 and 0.03% $CaCl_2 \cdot 2H_2O$ in ultrapure water. The pH was adjusted to 7.5 with Tris–HCl 1 M. Volumes of 250 ml were dispensed in 500 ml bottles and 1.5% unwashed agar (MP Biomedicals) were added. Bottles were placed in a water bath at 95 °C until complete dissolution of agar, then autoclaved. When cooled to about 60 °C, each bottle was supplemented with 1.25 ml autoclaved NH_4Cl 1 M, 0.50 ml autoclaved phosphate buffer at pH 7.5, 0.25 ml filter-sterilized trace element solution SL4 and 5 ml filter-sterilized sodium pyruvate 25% (Carl Roth GmbH, Karlsruhe, Germany). Phosphate buffer was prepared by adding appropriate volume of 0.5 M K_2HPO_4 to 30 ml of 0.5 M KH_2PO_4 until reaching pH 7.5. Solution SL4 was prepared by dissolving in ultrapure water acidified with a few drops of concentrated HCl 36 mg $MnCl_2 \cdot 4H_2O$, 44 mg $ZnSO_4 \cdot 7H_2O$, 230 mg $FeSO_4 \cdot 7H_2O$ and 5 mg $CuSO_4 \cdot 5H_2O$. After gentle hand-shaking, CDM medium was poured into petri dishes.

2.2.4. DBCM2 medium

DBCM2 medium preparation was modified from Dyall-Smith (2009). SW 25% was prepared by mixing 833 ml SW 30% with 167 ml ultrapure water. Volumes of 250 ml were dispensed into 500 ml bottles and 1.5% unwashed agar (MP Biomedicals) were added. The bottles were placed in a water bath at 95 °C until complete dissolution of agar, then autoclaved. 2.5 ml autoclaved Tris–HCl 1 M pH 7.4, 1.25 ml autoclaved NH_4Cl 1 M, 0.5 ml autoclaved phosphate buffer pH 7.4, 0.25 ml filter-sterilized SL10 solution and 1.1 ml filter-sterilized 25% sodium pyruvate were pipetted in each bottle. After gentle hand-shaking, DBCM2 medium was poured into petri dishes.

2.3. Plating

Three samples of 1.67 g were taken aseptically from different zones of a pack of commercial food-grade salt, placed in a single 50 ml tube and dissolved in 25 ml sterile SW 12% at 30 °C and 120 RPM until complete dissolution of salts (approximately 30 min). Plates were inoculated with 250 µl solution and sealed with plastic paraffin film. Incubation was performed at 37 °C for 5 weeks. For each food-grade salt and each medium, 3 replicates were analyzed with up to 3 dilutions. Dilutions with a number of colonies between 15 and 250 were kept for colony forming units (CFU) counting. Plates with less than 15 CFU without dilution were counted and plates with more than 250 CFU were re-inoculated with higher dilution. Counts are expressed as log10 (number of CFU). The sample arithmetic mean is calculated on the 3 replicates and the confidence interval is defined with a Student's *t*-value calculated with $n - 1$ degrees of freedom and $\alpha = 0.05$. Phenotypically distinct colonies were picked, streaked and stored at −80 °C in MGM 23% with glycerol (70:30).

2.4. Amplification of 16S rRNA gene from archaeal isolates and sequence analysis

Total DNA from haloarchaeal isolates was extracted by osmotic disruption in ultrapure water as follows: a small amount of colony from streaked plates was taken with a pipette tip, placed in 200 µl ultrapure water and vortex mixed for 1 min. In a PCR tube, 2 µl of this suspension was mixed with 18 µl PCR mix composed of Colorless GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$ (Promega), 200 µM deoxynucleoside triphosphates, 0.03 u/µl GoTaq® DNA polymerase (Promega), 0.25 mM forward primer S-D-Arch-0007-a-S-19 (5′-TTCCGGTTGATCCYGCCRG-3′) (Massana et al., 1997) and