The complete genome sequence of the archaeal isolate *Halomicrobium* sp. ZPS1 reveals the nitrogen metabolism characteristics under hypersaline conditions

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Abstract

**Purpose:** As a potential tool for the biodegradation of nitrogen contaminants, including nitrate, nitrite, and ammonium, in pickled foods with high salinity, the halophilic and denitrifying archaeal strain *Halomicrobium* sp. ZPS1 was isolated from edible salt particles.

**Methods:** Under anaerobic and static culture conditions, *Halomicrobium* sp. ZPS1 could simultaneously degrade nitrate, nitrite, and ammonium in liquid medium with 18% salinity and generate N₂O. To gain insight into these physiological characteristics, the complete genome of *Halomicrobium* sp. ZPS1 was sequenced to reveal the mechanism of nitrogen metabolism associated with salt-tolerance.

**Result:** The complete genome sequencing revealed a genome size of 3,094,203 bp with a circular chromosome and a GC content of 65.64%. Based on gene annotation, 3191 CDSs, 6 rRNA genes, and 76 tRNA genes were identified. Moreover, 28 genes were annotated as related to salt tolerance, ammonium assimilation, and a truncated denitrification pathway.

**Conclusion:** The annotated functional genes indicate that *Halomicrobium* sp. ZPS1 could be a candidate strain for the simultaneous removal of nitrate, nitrite, and ammonia in extremely high salt environments.

**Keywords:** *Halomicrobium* sp., Salt tolerance, Ammonia assimilation, Denitrification

Introduction

Various vegetables, which contain an abundance of nitrate and ammonium resulting from excessive nitrogen fertilization, can be pickled into various table food in China and other Asian countries. The nitrate contained in plant tissues can be reduced to nitrite, which causes anoxia poisoning and cancer (Zhong et al. 2002). Ammonium contained in plant tissues is also harmful to human health (Yusof et al. 2010). Therefore, the removal of excessive nitrate, nitrite, and ammonium is critical for the production of salted vegetables. Spoilage bacteria have been considered to reduce nitrate to nitrite in pickled vegetables, and lactic acid bacteria are considered to be powerful microbial barriers to the degradation of nitrite in pickled vegetables (Oh et al. 2004; Yan et al. 2008). For the removal of ammonium, some bacteria belonging to Planctomycetes are considered to completely oxidize ammonium to produce nitrogen through Anaammox processes (Jetten et al. 2001). However, the high salinity in salted vegetables inhibits the normal growth and metabolism of these microorganisms (Carr et al. 2002) and limits their capability of removing nitrogen contaminants.

Haloarapha, a group of halophilic archaea, could thrive in hypersaline environments from 1 M to 5.3 M NaCl (Aharon 2002; Pfeifer 2015), and it is widely
distributed on edible salt particles (Henriet et al. 2014). It has been reported that some haloarchaea, such as *Halofex mediterranei* (Cheung et al. 1997), *Halofex volcanii* and *Halofex denitrificans* (Torregrosa-Crespo et al. 2019), and *Halocrula marismortui* (Yoshimatsu et al. 2000), possesses denitrification genes and could perform denitrification or truncated denitrification pathways. Simultaneously, we found that haloarchaea can grow in medium with ammonium as the only nitrogen source (data not shown). Thus, we considered that haloarchaea could be a candidate microorganism for the simultaneous removal of nitrate, nitrite, and ammonia through assimilated and dissimilated nitrogen metabolism in a hypersaline environment. Here, the archaeal strain *Halomicrobium* sp. ZPS1 was isolated from edible salt particles produced in the Zhangpu Salt Field of China and was shown to be capable of degrading nitrate, nitrite, and ammonium under high salinity conditions. We describe the complete genome of *Halomicrobium* sp. ZPS1 to gain insight into the mechanism of nitrogen metabolism in extreme salt environments. The result may improve the theoretical basis and practical applications of haloarchaea in the removal of nitrogen during salted vegetable production, as well as other high salinity environments.

Materials and methods
The removal capability of nitrogen contaminants
*Halomicrobium* sp. ZPS1 was cultivated in HNM medium for 96 h, and 1 ml of culture was transferred to vials containing modified HNM medium, which contained 18% (w/v) NaCl and NaNO₃, NaNO₂, and NH₄Cl at a final concentration of 1 mM. The final pH was 7.0. After replacing the air with Ar, the vials were sealed with rubber septa and aluminum cap (Torregrosa-Crespo et al. 2019). During an observation period of 84 h, liquid and gas samples were collected every 12 h to determine the concentrations of nitrate, nitrite, ammonium, N₂O, and N₂. The concentration of nitrate and nitrite was measured by using cadmium column reduction and the N-(1-naphthyl)-ethylenediamine dihydrochloride spectrophotometric method (Ozdestan and Uren 2010; Ding et al. 2018). The concentration of ammonium was determined by the phenol-hypochlorous acid method (Ngo et al. 1982). Gas components were detected by gas chromatography with a TCD and ECD detector (SHI-MADZU-2014).

Sample preparation and genome DNA extraction
The strain *Halomicrobium* sp. ZPS1 was cultured in NOM liquid medium at 37 °C and enriched to the mid-logarithmic phase. We used the SDS method to extract the genomic DNA of *Halomicrobium* sp. ZPS1 and determined the quality and quantity of the DNA by agarose gel electrophoresis and Qubit 2.0, respectively.

Genome sequencing and assembly
The genome of *Halomicrobium* sp. ZPS1 was sent to Beijing Novogene Bioinformatics Technology Co., Ltd., and sequenced by Pacific Biosciences RS II single-molecule real-time (SMRT) sequencing technology and high throughput Illuminna sequencing technology (Mardis 2017; Hebert et al. 2018). We obtained low-quality reads after filtering raw data from the PacBio RS II and Illumina PE150 sequencer, and the reads were filtered by SMRT Link 5.0.1 ([https://www.pacb.com/support/software-downloads/](https://www.pacb.com/support/software-downloads/)) (Ardui et al. 2018; Reiner et al. 2018) to generate contigs.

Analysis of genome composition
We used GeneMarkS4.17 (Besemer et al. 2001) to predict protein-encoding genes, RNAmmer (Lagesen et al. 2007) to predict ribosomal RNA (rRNA) genes, tRNAscan-SE 1.3.1 (Lowe and Eddy 1997) to predict transfer RNA (tRNA) genes, and BLAST in the Rfam database to predict small nuclear RNAs (snRNA) (Gardner et al. 2008). The Genomics Islands were predicted through the IslandPath-DIOMB program (Hsiao et al. 2003), and the transposons were predicted through transposon PSI ([http://transposonpsi.sourceforge.net/](http://transposonpsi.sourceforge.net/)). PHAST (Zhou et al. 2011) was used for prophage prediction, and CRISPRFinder (Grisa et al. 2007) was used for CRISPR identification. The interspersed repetitive sequences were predicted through TRF (Tandem Repeats Finder, Version 4.07b) (Benson 1999).

Genome annotation
Five databases were used to predict gene functions: GO (Gene Ontology) (Ashburner et al. 2000), KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al. 2004; Kanehisa et al. 2006), COG (Clusters of Orthologous Groups) (Galperin et al. 2014), NR (Non-Redundant Protein Database databases) (Li et al. 2002), and CAZy (Carbohydrate-Active enzyme database) (Cantarel et al. 2008). A whole-genome Blast search, which had an E value of less than 1e−5 and a minimal alignment length percentage larger than 40%, was performed against above the five databases. In addition, we used Mega7.0, and DNAMAN software to analyze sequences of 16S rRNA and enzymes.

The assessment of potential secondary metabolites
To assess the potential secondary metabolites of *Halomicrobium* sp. ZPS1, we used antiSMASH (Medema et al. 2011) to predict the number of biosynthetic gene clusters (BGCs).

Data deposition
The complete genome sequence of *Halomicrobium* sp. ZPS1 has been deposited in GenBank under the accession number CP045142.
Results

Nitrogen metabolism pathway of *Halomicrobium* sp. ZPS1 under high salinity conditions

Under the condition of 18% salt concentration, the degradation rate of NO$_3^-$ in the medium reached 95.1% in 60 h, and the degradation rate of NO$_2^-$ reached 98.3% in 84 h after fluctuating. NH$_4^+$ continued to decline, and the degradation rate reached 52.2% at 84 h. Gas detection results showed that a large amount of N$_2$O was generated without N$_2$ production. These results suggest that the strain *Halomicrobium* sp. ZPS1 has the ability to simultaneously metabolize NO$_3^-$, NO$_2^-$, and NH$_4^+$ to N$_2$O under high salt and anaerobic conditions (Fig. 1).

General genome features of *Halomicrobium* sp. ZPS1

After filtering the raw data, we obtained clean data and 108,469 reads with 1,028,063,020 nucleotides, and the mean read length was 9477 bp. Then, the raw data were assembled through SMRT Link v5.0.1, generating one contig. We obtained a complete genome sequence of *Halomicrobium* sp. ZPS1, which contained a circular chromosome of 3,094,203 bp in length and a G+C content of 65.64%. Meanwhile, 6 rRNA operons, 76 tRNA genes, 10 genomics islands, 1 CRISPR system, 2 prophages, and 3191 protein-coding genes (CDSs) were identified (Fig. 2 and Table 1). The results of genome annotation showed that 1426 CDSs could be annotated by the KEGG database, 2212 CDSs could be annotated by the COG database, and 1967 CDSs could be annotated by the GO database.

Functional annotation of *Halomicrobium* sp. ZPS1

To identify the gene functions of *Halomicrobium* sp. ZPS1, the genome was functionally annotated with five databases. The GO classification results revealed that the

![Fig. 1 Degradation of nitrate, nitrite, and ammonium by *Halomicrobium* sp. ZPS1 at NaCl concentration of 18%](image)

The mechanism of salt tolerance

In addition, through COG annotation of the complete genome sequence of *Halomicrobium* sp. ZPS1, 13 genes related to Na$^+$/K$^+$ transportation were also found, which means that *Halomicrobium* sp. ZPS1 could exist in saline or hypersaline environments through the enrichment of potassium ions and discharge of sodium ions, also called the “salt-in” strategy (Table 2). *Halomicrobium* sp. ZPS1 balanced the osmotic pressure of the medium by accumulating intracellular K$^+$ to a high concentration through a K$^+$ transport system and pumping Na$^+$ through an electrogenic Na$^+$/H$^+$ antiporter (Mnh). Moreover, the Na$^+$/H$^+$ antiporter also plays an essential role in the homeostasis of intracellular pH (Aharon 1999).

Genes involved in nitrogen metabolism

By comparing the amino acid sequence of *Halomicrobium* sp. ZPS1 with the COG database, we obtained a total of 2213 genes encoding different functional enzymes, and at least 10 reductase genes involved in nitrogen metabolism were obtained, including nitrate reductase-, nitrite reductase-, nitric oxide reductase-, nitrate/nitrite transporter-, nitrous oxide reductase-, glutamate dehydrogenase-, glutamate
synthase- and ammonia channel protein-encoding genes (Table 2). However, through the comparison with other denitrifiers, we found that the gene encoding the nitrous oxide reductase in *Halomicrobium* sp. ZPS1 was *nosL*, which might bear a functional homologous gene. This gene did not encode a protein with synthetase nitrous oxide reductase (Lycus et al. 2017). We consider that *Halomicrobium* sp. ZPS1 could perform a truncated denitrification pathway, reducing nitrate or nitrite to nitrous oxide.

Based on the analysis of the KEGG database, the nitrogen metabolism pathways are performed (Fig. 4), and *Halomicrobium* sp. ZPS1 could reduce nitrate or nitrite to nitrous oxide through truncated denitrification under salty environments. Nitrate is first reduced to nitrite by nitrate reductase, then to nitric oxide by nitrite reductase, and finally to nitrous oxide by nitric oxide reductase. In the assimilatory nitrate reduction pathway, nitrite existing in salted vegetables could be transported into the cells through nitrate/nitrite transporters and further reduced to $\text{NH}_4^+$ by assimilatory nitrite reductase.

**Discussion**

Haloarchaea exist in saline or hypersaline environments where other microorganisms hardly exist, and they could

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**Table 1** Overview of genomic features of *Halomicrobium* sp. ZPS1

| Attributes                        | Characteristics |
|-----------------------------------|-----------------|
| Genome size (Mb)                  | 3.09            |
| GC content (%)                    | 65.64           |
| CDS                               | 3191            |
| tRNA genes                        | 76              |
| rRNA genes                        | 6               |
| snRNA genes                       | 0               |
| Secondary metabolite BGCs         | 3               |
| Genes assigned to COG             | 2212            |
| Genes assigned to KEGG            | 1426            |
| Genes assigned to GO              | 1967            |
| Genes assigned to CAZy            | 47              |
| Genes assigned to NR              | 1165            |
| GenBank accession number          | CP045142        |
maintain normal growth and metabolism in these environments. It has been found that the intracellular ionic composition of haloarchaea is different from the outer extracellular environment; the environment contains NaCl as the main salt, while the cytoplasm contains more KCl than outside environment (Aharon 1999). This could be regarded as a salt-in strategy, which is expected to provide the ability to survive in high salt environments. In this study, we annotated genes related to the K⁺ transport system and Na⁺/H⁺ antiporter (Mnh), which indicates that Halomicrobium sp. ZPS1 grows in high salinity environments through a “salt-in” strategy, and K⁺ existing in the medium is critical for the growth and metabolism of Halomicrobium sp. ZPS1.

Under the condition of 18% salt concentration, the growth and metabolism of Halomicrobium sp. ZPS1, the concentration of NO₃⁻, NO₂⁻, and NH₄⁺ could be decreased, and N₂O was simultaneously produced; however, no N₂ was produced. Based on these physiological characteristics, we can speculate that the degradation of nitrate and nitrite is attributed to truncated denitrification.

Denitrification in microorganisms is a dissimilatory process in which nitrate or nitrite is reduced to nitrogenous gas (Wei et al. 2015); this process includes four steps, NO₃⁻→NO₂⁻→NO→N₂O→N₂ (Verstraete and Focht 1977), and these four steps are catalyzed by four different kinds of enzymes, nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (Philippot 2002). Through the annotation of the complete genome of Halomicrobium sp. ZPS1, the nar, nir, and nor were found but nos was not, which means that ZPS1 could perform a truncated denitrification pathway, and nitrate and nitrite could be reduced to nitrous oxide and released to the atmosphere, making salted vegetables free of nitrate or nitrite residues.

Moreover, according to the previous physiological characteristics, we can also speculate that the degradation of ammonium is achieved by ammonium assimilation. Through the annotation of the complete genome of Halomicrobium sp. ZPS1, genes related to ammonium assimilation and ammonia transportation, including glutamine synthetase-, glutamate dehydrogenase-, and
Table 2 The denitrification related genes in COG database

| Identity | E value | Functional description                                      | Functional class                                                                 | Class description                                                                 |
|----------|---------|------------------------------------------------------------|----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| GM001562 | 0.00E+00 | Nitrate reductase gamma subunit                            | CP                                                                               | Energy production and conversion                                                   |
| GM002221 | 1.40E−96 | Nitrate/nitrite transporter NarK                           | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM003113 | 1.00E−223| Nitrate reductase beta subunit                             | CP                                                                               | Energy production and conversion                                                   |
| GM003113 | 1.00E−223| Nitrate reductase beta subunit                             | CP                                                                               | Energy production and conversion                                                   |
| GM003114 | 0.00E+00 | Nitrate reductase alpha subunit                            | CP                                                                               | Energy production and conversion                                                   |
| GM000464 | 6.60E−102| Nitros oxide reductase accessory protein NosL              | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000538 | 9.60E−77 | Ferredoxin subunit of nitrate reductase or a ring-hydroxylating dioxygenase | PQ                                                                               | Inorganic ion transport and metabolism                                             |
| GM001048 | 7.90E−97 | Ferredoxin subunit of nitrate reductase or a ring-hydroxylating dioxygenase | PQ                                                                               | Inorganic ion transport and metabolism                                             |
| GM002611 | 0.00E+00 | Nitric oxide reductase large subunit                       | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000159 | 5.20E−07 | Glutamine synthetase                                       | E                                                                                | Amino acid transport and metabolism                                                |
| GM000568 | 9.60E−77 | Glutamate dehydrogenase/leucine dehydrogenase             | E                                                                                | Amino acid transport and metabolism                                                |
| GM000588 | 6.00E−239| Glutamate dehydrogenase/leucine dehydrogenase             | E                                                                                | Amino acid transport and metabolism                                                |
| GM000589 | 0.00E+00 | Glutamate synthase domain 3                                | E                                                                                | Amino acid transport and metabolism                                                |
| GM001552 | 1.50E−253| Ammonia channel protein AmtB                               | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000021 | 2.50E−251| Na⁺-dependent transporter, SNF family                      | R                                                                                | General function prediction only                                                    |
| GM000159 | 7.80E−187| ABC-type Na⁺ efflux pump, permease component               | CP                                                                               | Energy production and conversion                                                   |
| GM000311 | 1.30E−180| Multisubunit Na⁺/H⁺ antiporter, MnhE subunit              | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000312 | 1.30E−42 | Multisubunit Na⁺/H⁺ antiporter, MnhF subunit              | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000313 | 1.30E−42 | Multisubunit Na⁺/H⁺ antiporter, MnhG subunit              | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000315 | 7.20E−81 | Multisubunit Na⁺/H⁺ antiporter, MnhB subunit              | P                                                                                | Inorganic ion transport and metabolism                                             |
| GM000316 | 3.50E−59 | Multisubunit Na⁺/H⁺ antiporter, MnhC subunit              | P                                                                                | Inorganic ion transport and metabolism                                             |
ammonia channel protein-encoding genes, were also found in the complete genome. This means that ammonia could be transported into the cytoplasm from the periplasm through the ammonia channel protein (amtB) and further assimilated to L-glutamate from L-glutamine (Fig. 4), which could maintain the normal growth of *Halomicrobium* sp. ZPS1. This means that NH$_4^+$ produced from nitrite or that existed in the vegetables could be involved in carbon metabolism by the glutamine synthetase/glutamate synthase pathway, and maintain the growth of *Halomicrobium* sp. ZPS1 without additional carbon and nitrogen sources, and it makes it possible to eliminate nitrate, nitrite, and ammonia simultaneously.

Oxalic, malate, malonic, erythorbic acid, glutamic acid, and other organic acids are present in radish (Gutiérrez and Perez 2004). Genes involved in the reductive tricarboxylic acid (TCA) cycle were obtained through the KEGG database except 2-oxoglutarate dehydrogenase-encoding genes, which means that malate could act as an electron donor and carbon source for cells, allowing the cells to maintain growth. The energy for *Halomicrobium* sp. ZPS1 to synthesize fumarate and succinate

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**Table 2** The denitrification related genes in COG database (Continued)

| Identity (%) | E value | COG gene ID | Functional description | Functional class | Class description |
|--------------|---------|-------------|------------------------|------------------|-------------------|
| ZPS1 GM000318 | 100 | 0.00E+00 | YP 003178676 | Formate hydrogenlyase subunit 3/Multisubunit Na$^+/H^+$ antiporter, MnHd subunit | CP | Energy production and conversion Inorganic ion transport and metabolism |
| ZPS1 GM000779 | 100 | 1.10E−262 | YP 003175955 | Na$^+$-dependent transporter, SNF family | R | General function prediction only |
| ZPS1 GM001320 | 100 | 4.10E−279 | YP 003176524 | Na$^+$-driven multidrug efflux pump | V | Defense mechanisms |
| ZPS1 GM003089 | 100 | 2.20E−158 | YP 003178260 | Predicted Na+-dependent transporter | R | General function prediction only |
| ZPS1 GM001370 | 100 | 0.00E+00 | YP 003176577 | Trk K$^+$ transport system, NAD-binding component | P | Inorganic ion transport and metabolism |
| ZPS1 GM002514 | 99.8 | 0.00E+00 | YP 003177697 | Trk-type K$^+$ transport system, membrane component | P | Inorganic ion transport and metabolism |

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**Fig. 4** Genome-based nitrogen metabolism pathways of *Halomicrobium* sp. ZPS1. Nitrate and nitrite could be reduced to nitrous oxide through a truncated denitrification pathway. Ammonium existing in the vegetables or formed by nitrite assimilatory could be transported into the cytochrome and transformed to L-glutamate, formed L-glutamate, which could participate in glutamate metabolism. *Halomicrobium* sp. ZPS1 could perform a reductive TCA cycle, and electrons generated from succinate to fumarate could be transferred to denitrification pathways. NarG, nitrite reductase; NirK, cooper-containing nitrite reductase; Nor, nitric oxide reductase; NirA, ferredoxin-nitrite reductase; Amt, ammonia channel protein; glnA, glutamine synthetase; GLT1, glutamate synthase; gdhA, glutamate dehydrogenase.

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comes from metabolizing the malate in salted vegetables, and the transformation of fumarate and succinate provides electrons for the truncated denitrification pathway (Fig. 4). Thus, nitrate, nitrite, and ammonium could act as nitrogen resources, and organic acids present in salted vegetables act as carbon resources to provide electrons and energy for cells; therefore, the cells are able to maintain respiration and growth and remove nitrogen contaminants without additional carbon and nitrogen resources.

The safety of haloarchaea applications for food has not been confirmed so far, mainly because it is impossible to simulate the high salt environment in which that haloarchaeabite inhabits in mice. Edible sea salt and mineral salt contain a large amount of haloarchaea; however, cells could rupture and die due to the osmotic pressure of the normal human body; therefore, it is difficult to find haloarchaea in the human body. If the safety of haloarchaea can be confirmed, they can provide a feasible solution for the complete removal of nitrogen contaminants from high-salt vegetables. *Halomicrobium* sp. ZPS1 could be a potential resource for the elimination of nitrite and nitrate in salted food.

**Conclusion**

This study reports the complete genome sequence of *Halomicrobium* sp. ZPS1 isolated from a salt mine in China. The phylogeny analysis indicates that ZPS1 is closely related to *Halomicrobium mukohatae*i. By assembling and annotating the complete genome, we considered that ZPS1 could grow in a high salinity environment by using a "salt-in" strategy and ammonium assimilation and reduce nitrate into N₂O via a truncated denitrification pathway. The results indicated that *Halomicrobium* sp. ZPS1 might be a candidate strain for the simultaneous removal of nitrate, nitrite, and ammonia in the process of salted vegetable production, as well as in other high salinity environments.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01575-8.

**Additional file 1: Figure S1.** GO classification of genome function in *Halomicrobium* sp. ZPS1. **Figure S2.** KEGG categories of coding proteins in *Halomicrobium* sp. ZPS1. **Figure S3.** Phylogenetic tree based on 16s rRNA sequences of *Halomicrobium* sp. ZPS1. Bootstrap values greater than 50% are shown. Scale bar represents substitutions per nucleotide.

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**Competing interests**

The authors declare that they have no competing interests.

**Research involving human participants and/or animals**

N/A

**Informed consent**

N/A

**Authors’ contributions**

All the authors designed this study. XYH performed the experiment and drafted the manuscript. XYH, CIZ, KJ, KS and LZ analyzed and performed the data. ZSH, YW and QX contributed reagents/materials/analysis tools. The author(s) read and approved the final manuscript.

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