

Genomic Analysis of the Extremely Halophilic Archaeon *Halobacterium noricense* CBA1132 Isolated from Solar Salt That Is an Essential Material for Fermented Foods

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The extremely halophilic archaeon *Halobacterium noricense* is a member of the genus *Halobacterium*. Strain CBA1132 (= KCCM 43183, JCM 31150) was isolated from solar salt. The genome of strain CBA1132 assembled with 4 contigs, including three rRNA genes, 44 tRNA genes, and 3,208 open reading frames. Strain CBA1132 had nine putative CRISPRs and the genome contained genes encoding metal resistance determinants: copper-translocating P-type ATPase (CtpA), arsenical pump-driving ATPase (ArsA), arsenate reductase (ArsC), and arsenical resistance operon repressor (ArsR). Strain CBA1132 was related to *Halobacterium noricense*, with 99.2% 16S rRNA gene sequence similarity. Based on the comparative genomic analysis, strain CBA1132 has distinctly evolved; moreover, essential genes related to nitrogen metabolism were only detected in the genome of strain CBA1132 among the reported genomes in the genus *Halobacterium*. This genome sequence of *Halobacterium noricense* CBA1132 may be of use in future molecular biological studies.

Keywords: Haloarchaea, extremely halophilic archaea, *Halobacterium*, genomic analysis

Introduction

Microorganisms that grow in extreme conditions, or extremophiles, have been an important source of stable and valuable enzymes with novel activities and applications because the majority of extremophiles are members of Archaea that have novel metabolic pathways [18]. Halophiles are found in environments with high salt concentrations, and can be classified as slightly, moderately, or extremely halophilic based on their requirement for sodium chloride [10]. Many studies have showed that halophiles are also

found in the fermented foods, using culture-dependent and culture-independent methods [1, 7, 19, 22, 32–37]. Solar salt has been widely added to the Korean traditional fermented foods, such as salted fish, soybean paste, kimchi (fermented vegetables), and jeotgal (salted fermented seafoods). Several novel strains affiliated to extremely halophilic archaea have been isolated from solar salt samples [5, 6, 11, 19, 24–26, 28, 30, 39–42, 47–49].

The genus *Halobacterium* (*Hbt.*) belongs to the class Halobacteria and was first proposed by Elazari-Volcani [12] for the species *Halobacterium cutirubrum*, which was

later renamed as *Halobacterium salinarum* [46]. *Halobacterium* species have been isolated from salt mines [16], fermented fish [27], saline lakes [50], and marine solar salterns [17]. Among the members in the genus *Halobacterium*, *Hbt. noricense* A1^T was isolated from a salt mine at Altaussee, Austria. Strain A1^T requires at least 12.5% NaCl and 0.6 M MgCl₂ for growth and shows optimal growth with 15.0–17.5% of NaCl and 0.7–0.8 M of MgCl₂ [16]. At the time of writing, the *Halobacterium* genus currently comprises of five species [31] and the genomes of only three strains (*Hbt.* sp. DL1, *Hbt. salinarum* NRC-1, and *Hbt. salinarum* R1) have been reported (GOLD, <https://gold.jgi.doe.gov>). In this work, the genomic features of *Halobacterium* strain CBA1132 that was isolated from solar salt are described. The genomic information of strain CBA1132 may improve the understanding of haloarchaeal characteristics and useful halophilic enzymes.

Materials and Methods

Sample Collection and Archaeal Strain Isolation

The extremely halophilic archaeon CBA1132 was isolated from solar salt in South Korea (36.5875 N, 126.3745 E) and cultivated on JCM medium No. 574 (containing per liter distilled water, pH 7.5, 833 ml MDS salt water, 1 ml FeCl₂ solution, 1 ml trace element solution, 0.25 g peptone (Oxoid), 0.05 g yeast extract (Difco), 5 ml 1 M NH₄Cl, 2 ml potassium phosphate buffer, 3 ml vitamin solution, and 10 ml 1 M sodium pyruvate solution) at 37°C for 2 months. To obtain pure cultures, a single colony of strain CBA1132 was repeatedly transferred to the fresh JCM medium No. 574 more than three times. The cell morphology of CBA1132 and examination for contamination by other microorganisms were detected using a light microscope (ECLIPSE 80i; Nikon). The strain has been deposited at the Korean Culture Center of Microorganisms (KCCM) and Japan Collection of Microorganisms (JCM) under accession number KCCM 43183 and JCM 31150, respectively.

Genomic DNA Extraction and Whole Genome Sequencing

Cells were incubated under aerobic conditions at 37°C for 2 weeks to extract genomic DNA. Genomic DNA was extracted using a QuickGene DNA tissue kit S (Kurabo) and purified using an MG Genomic DNA purification kit (Doctor Protein) according to the manufacturers' instructions. Genomic DNA of strain CBA1132 was sequenced using next-generation sequencing technology (PacBio RS II system; Pacific Biosciences) according to the manufacturer's instructions and was assembled using PacBio SMRT Analysis 2.3.0 (Pacific Biosciences). The genome sequence of strain CBA1132 was deposited in the DDBJ under the accession numbers BCMZ01000001–BCMZ01000004. The project information according to the minimum information about a genome sequence

Table 1. Genome and environmental features of *Halobacterium noricense* CBA1132 according to the MIGS recommendations.

Item	Description
MIGS data	
Investigation_type	Bacteria_archaea
Project_name	Genome sequencing of <i>Halobacterium</i> sp. CBA1132
Collected_by	Seong Woon Roh
Collection_date	2015
Lat_lon	36.5875 N 126.3745 E
Depth	0 m
Alt_elev	NA
Country	South Korea
Environment	Solar salt
Ref_biomaterial	NA
Biotic_relationship	Free living
Trophic_level	Heterotroph
Rel_to_oxygen	Aerobe
Isol_growth_condt	NA
Sequencing_meth	PacBio
Num_replicons	NA
Assembly	PacBio SMRT Analysis 2.3.0
Finishing_strategy	Draft
Annot_source	CLgenomics, RAST server
Estimated_size	3,012,807 bp
Biome	ENVO:00000569
Feature	ENVO:00000055
Material	ENVO:00002002
Geo_loc_name	South Korea
Sample-material	Solar salt
Source_mat_id	KCCM 43183, JCM 31150
Genome assembly data	
Assembly method	PacBio SMRT Analysis 2.3.0
Assembly name	CBA1132
Genome coverage	300×
Sequencing technology	PacBio RS II system

(MIGS) recommendation [14] is shown in Table 1.

Phylogenetic Analysis

For phylogenetic analysis, three phylogenetic trees were constructed based on the 16S rRNA gene, five housekeeping genes for multilocus sequence typing (MLST), and average nucleotide identity (ANI). The 16S rRNA gene sequence of strain CBA1132 was extracted from the genome sequences using the RNAmmer 1.21 server [21]. The 16S rRNA sequence of strain CBA1132 was

Table 2. General features of the *Halobacterium noricense* CBA1132 and other *Halobacterium* genomes.

	Genome size (bp)	rRNA	tRNA	Coding sequence	G + C content (%)	ANI value (%)
<i>Halobacterium noricense</i> CBA1132	3,012,807	3	44	3,084	65.95	-
<i>Halobacterium</i> sp. DL1	3,162,560	3	46	3,280	66.4	79.71
<i>Halobacterium salinarum</i> NRC-1	2,571,010	4	47	2,622	65.9	76.91
<i>Halobacterium salinarum</i> R1	2,668,776	3	47	2,749	65.7	77.18

compared with those of closely related strains using EzBioCloud (<http://www.ezbiocloud.net>). Five different housekeeping genes were used to generate the MLST phylogenetic tree: V-type ATP synthase subunit B (*atpB*), elongation factor 2 (*EF-2*), DNA repair and recombination protein RadA (*radA*), DNA-directed RNA polymerase subunit beta (*rpoB*), and protein translocase subunit SecY (*secY*). The 16S rRNA gene sequences and concatenated amino acid sequences inferred from the five genes for MLST were aligned with those of the most closely related species, respectively, using the multiple alignment program ClustalW [45]. Phylogenetic relationships between strain CBA1132 and its most closely representative species were determined using the MEGA6 software [44]. Phylogenetic consensus trees were constructed using the neighbor-joining (NJ) [38], maximum-parsimony (MP) [20], and maximum-likelihood (ML) [13] methods with 1,000 randomly selected bootstrap replicates. The phylogenetic tree based on ANI values was constructed using CLgenomics ver. 1.52 by ChunLab Inc. (<http://www.chunlab.com/genomics>).

Genomic Analysis

Gene annotation was performed using the RAST server [2]. The rRNA and tRNA genes were identified using the RNAmmer 1.21 server [21] and tRNA scan-SE 1.21 [23], respectively. Classification analysis for the SEED subsystem was performed on genome data using the RAST server [2]. Clustered regularly interspaced short palindromic repeat (CRISPR) arrays were analyzed using the CRISPR finder program [15]. To evaluate similarity between genome sequences, ANI values were analyzed between strain CBA1132 and the reported genomes in the genus *Halobacterium* using EzBioCloud as described by Moon *et al.* [29]. The genome of strain CBA1132 was aligned with other genomes in the genus *Halobacterium* using the Mauve alignment 2.4.0 with default setting for visualizing whole genome alignments [9].

Results and Discussion

Colonies of strain CBA1132 were circular and pink in colour, and coccoid form of the cells was observed. The draft genome sequence of strain CBA1132 comprised of four contigs, with a genome size of 3,012,807 bp and an N50 of 2,587,453 bp. As summarized in Table 2 and visualized in Fig. 1, the genome contained 3,208 open reading frames (ORFs), 3,084 coding sequences (CDS), three

rRNA genes (5S, 16S, and 23S), and 44 tRNA genes. The genomic G + C content was 65.95%. A total of 2,536 genes were analyzed based on the COG functional categories (<http://www.ncbi.nlm.nih.gov/COG/>): 159 genes in energy production, 72 in carbohydrate transport and metabolism, 213 in amino acid transport and metabolism, 62 in nucleotide transport and metabolism, and 99 in coenzyme transport and metabolism (Table 3). Based on the RAST pipeline, metal resistance-associated genes coding copper-translocating P-type ATPase (*CtpA*), arsenical pump-driving ATPase (*ArsA*), arsenate reductase (*ArsC*), and arsenical

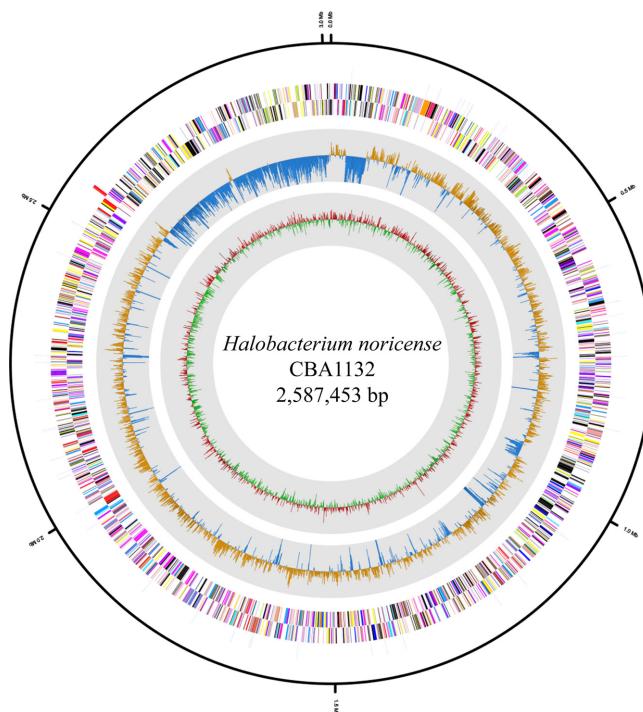


Fig. 1. Graphic circular map of the *Halobacterium noricense* CBA1132 genome. Antisense and sense strands (colored according to COG categories) and RNA genes (red, tRNA; blue, rRNA) are shown from the outer periphery to the center. Inner circles show the GC skew, with yellow and blue indicating positive and negative values, respectively, and the GC content is indicated in red and green. This genome map was visualized using CLgenomics 1.52 (Chun Lab Inc.).

Table 3. Genes associated with general COG functional categories in the genome of *Halobacterium noricense* CBA1132.

COG	Description of function	Number of genes	Ratio
J	Translation, ribosomal structure, and biogenesis	151	5.95%
K	Transcription	160	6.31%
L	Replication, recombination, and repair	158	6.23%
D	Cell cycle control, cell division, chromosome partitioning	27	1.06%
O	Posttranslational modification, protein turnover, chaperones	90	3.55%
M	Cell wall/membrane/envelope biogenesis	48	1.89%
N	Cell motility	23	0.91%
P	Inorganic ion transport and metabolism	159	6.27%
T	Signal transduction mechanisms	87	3.43%
C	Energy production and conversion	159	6.27%
G	Carbohydrate transport and metabolism	72	2.84%
E	Amino acid transport and metabolism	213	8.40%
F	Nucleotide transport and metabolism	62	2.44%
H	Coenzyme transport and metabolism	99	3.90%
I	Lipid transport and metabolism	67	2.64%
Q	Secondary metabolites biosynthesis, transport, and catabolism	38	1.50%
R	General function prediction only	236	9.31%
S	Function unknown	687	27.09%
Total		2,536	100.00%

resistance operon repressor (*ArsR*) were annotated. The *ars* operon is required for the detoxification of arsenate and arsenite. This operon is also found in strain NRC-1 [16].

Strain CBA1132 was most closely related to *Hbt. noricense* A1^T (99.3% of 16S rRNA gene sequence similarity), *Hbt. jilantaiense* NG4^T (97.4%), *Hbt. salinarum* JCM 8978^T (97.3%), *Hbt. rubrum* TGN-42-S1^T (97.0%), and *Hbt. piscisalsi* HPC1-2^T (96.3%). Phylogenetic analysis showed that strain CBA1132 clustered with species of the genus *Halobacterium* and was closely associated with *Hbt. noricense* A1^T (Fig. 2A). In the genus *Halobacterium* clade, each branch was conserved on the basis of not only NJ algorithm but also MP and ML algorithms. Moreover, high bootstrap values indicated that strain CBA1132 belongs to the genus *Halobacterium*. The phylogenetic trees based on MLST genes and ANI with the three reported strains in genus *Halobacterium* (*Hbt.* sp. DL1, *Hbt. salinarum* NRC-1, and *Hbt. salinarum* R1) shown in Figs. 2B and 2C indicated that strain CBA1132 and strain DL1 cluster separately to strains NRC-1 and R1, suggesting that strains CBA1132 and DL1 are more closely related than the other two strains.

Strain CBA1132 had no confirmed CRISPRs with nine putative CRISPRs, although *Hbt.* sp. DL1, *Hbt. salinarum*

NRC-1, and *Hbt. salinarum* R1 had 2, 0, and 0 confirmed CRISPRs, respectively. Strain CBA1132 showed 79.71% (79.53% in reciprocal), 76.91% (76.97%), and 77.18% (77.39%) ANI value with *Hbt.* sp. DL1, *Hbt. salinarum* NRC-1, and *Hbt. salinarum* R1, respectively. This result showed that strain CBA1132 has evolutionary distinctness compared with *Hbt.* sp. DL1, *Hbt. salinarum* NRC-1, and *Hbt. salinarum* R1. The result of the whole genome alignment using Mauve, represented by the highly homologous regions with the locally collinear blocks, suggested that strain CBA1132 shows more genome rearrangements as compared with the other three genomes of genus *Halobacterium* (Fig. 3). Based on the ORF-independent comparison (ORF-IDC) method by ChunLab Inc. [8], a total of six genes related to nitrogen metabolism, such as MFS transporter, cytochrome B, nitrate reductase, and nitrous oxide reductase accessory protein, were only found in the genome of strain CBA1132, but not in other reported genomes in genus *Halobacterium*. Moreover, through the KEGG pathway, the genes related to dissimilatory nitrate reduction, assimilatory nitrogen reduction, and denitrification were detected in the genome of strain CBA1132.

The extremely halophilic archaea can survive in high metal

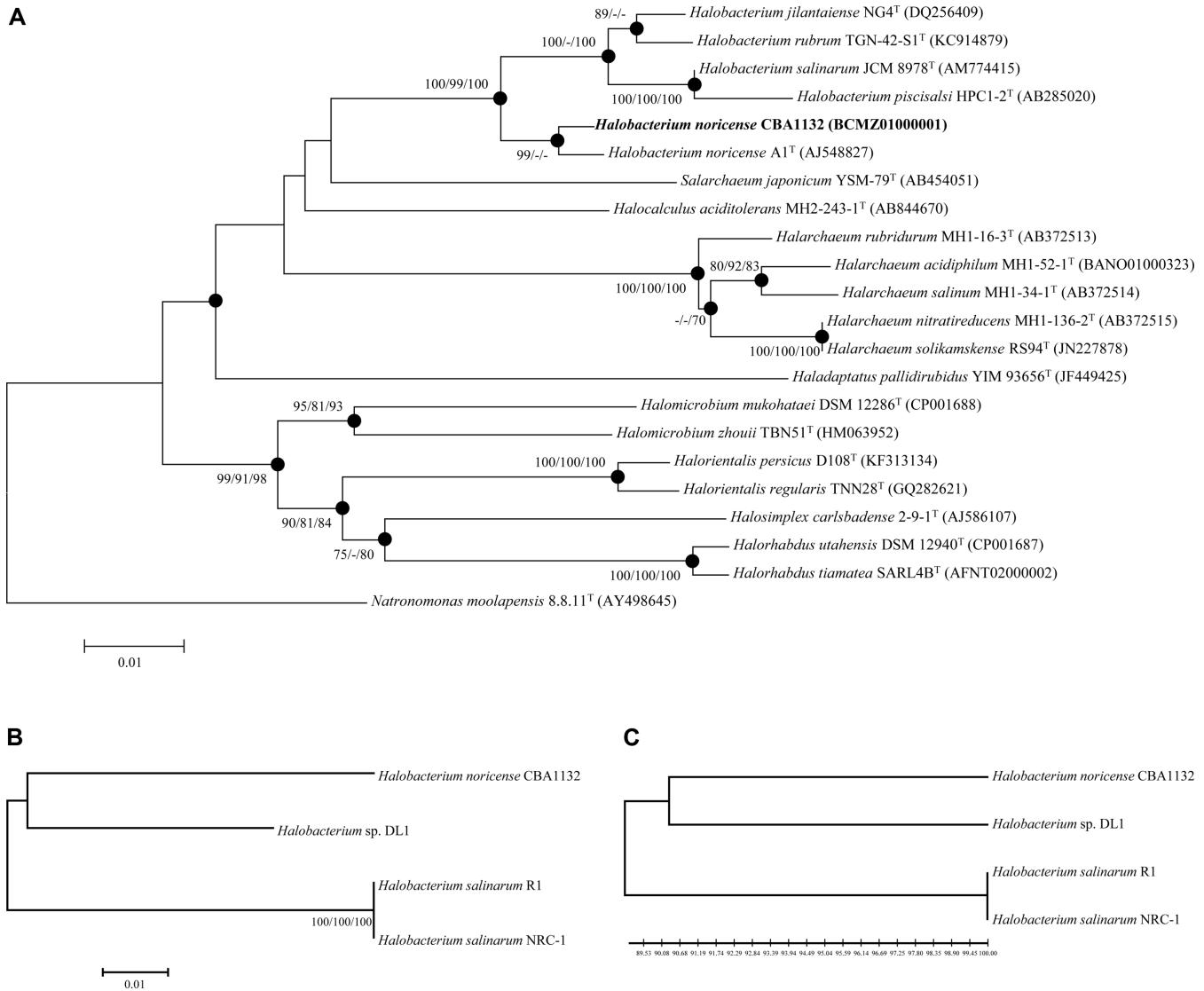


Fig. 2. Phylogenetic consensus trees based on 16S rRNA gene, MLST genes, and ANI.

(A) Neighbor-joining (NJ) algorithm for the 16S rRNA gene sequence showing the taxonomic position of strain CBA1132. The closed circles indicate the nodes were identified using both the maximum-parsimony (MP) and maximum-likelihood (ML) algorithms. The numbers at nodes represent bootstrap values (>70%), calculated using NJ/ME/ML probabilities based on 1,000 replicates. *Natronomonas moolapensis* 8.8.11^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide. (B) Phylogenetic tree based on MLST genes. Amino acid sequences of *atpB*, *EF-2*, *radA*, *rpoB'*, and *secY* were analyzed using ML algorithm. (C) ANI phylogenetic tree. Using CLgenomics, the phylogenetic tree was constructed based on ANI values.

concentration through the mechanism of metal resistance [43]. Through the gene prediction processes, metal resistance-associated genes were found in the genome of strain CBA1132. These results indicate that strain CBA1132 might be able to survive at high concentrations of metal ions. Nitrogen is an essential and major element in all organisms; the nitrogen-cycle metabolic pathway has been studied widely in Bacteria, Eukarya, and Archaea [3, 4]. However,

nitrate reduction and denitrification-related genes were found only in the genome of strain CBA1132 among genus *Halobacterium*. The genomic analysis of strain CBA1132 and the whole genome alignments in genus *Halobacterium* would increase our knowledge of the extremely halophilic archaea. Further studies of the genome sequence of *Hbt. noricense* CBA1132 will be useful for potential commercial applications, using the haloarchaeal extremozymes and their physiological

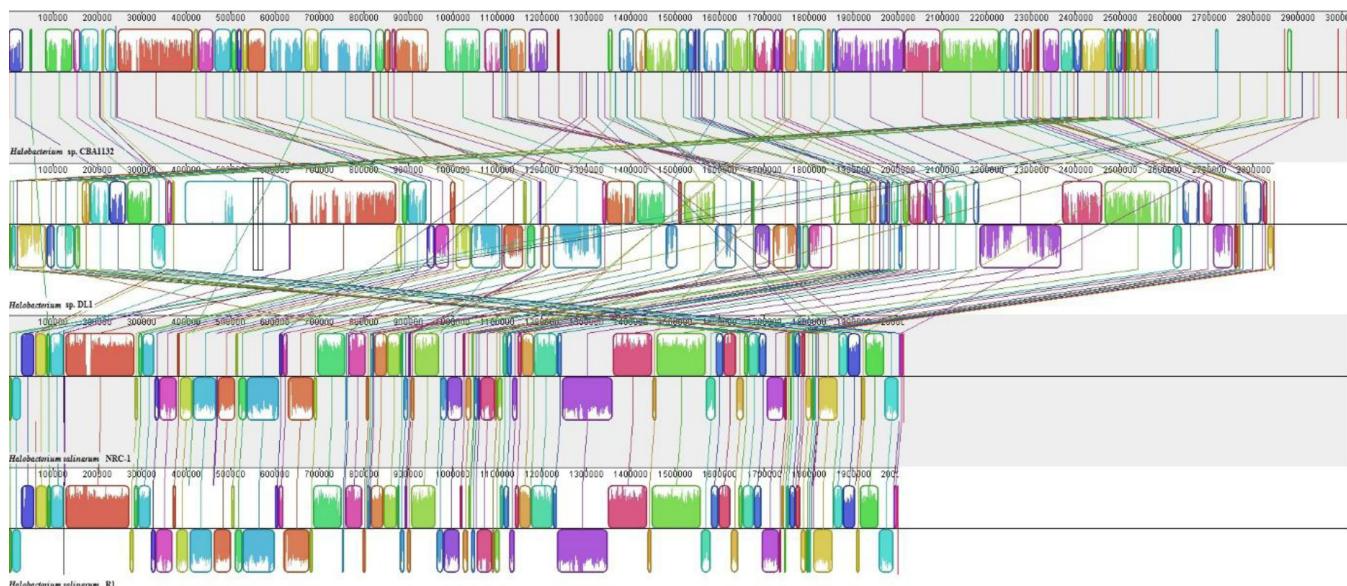


Fig. 3. Mauve alignment of the genome of *Halobacterium noricense* CBA1132 and genomes of *Hbt. sp. DL1*, *Hbt. salinarum* NRC-1, and *Hbt. salinarum* R1.

The locally collinear blocks (LCBs) represent the highly homologous regions and are shown with identical colors. LCBs indicated below the horizontal black line represent reverse complements of the LCBs of the strain CBA1132 reference genome. The genomes were drawn to scale based on the strain CBA1132 genome.

functionality, and for future molecular biological studies.

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