Characterization of Plant-Growth Promoting Archaea Associated with the Rhizosphere of Salsola stocksii and Atriplex a

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Abstract

Halophilic microorganisms play a crucial role in plant health and growth in salinity affected lands. The objective of this study was to evaluate the haloarchaeal diversity from the rhizosphere of halophytes and plant growth promoting abilities of these strains. The whole genome sequences of two haloarchaeal strains, Halorubrum lacusprofundi HL1RP11 and Halobacterium noricense NRS2HaP9, were analyzed, and genes related to plant growth promoting traits were identified. Phylogenetic analysis showed that archaeal strains of Halococcus, Halorubrum, Halobacterium and Natrinema were dominant in the rhizosphere of halophytes. More than 60% of the strains were positive for phosphate solubilization and IAA production. About 50% of strains were positive for nitrogen fixation, while 33% were siderophore producers. More than 40% of haloarchaeal strains showed the heavy metal resistance for Nickel, Cadmium, Chromium and Zinc at a concentration of 5 mM. Genes involved in plant growth promotion were identified through annotation. Gene clusters related to secondary metabolites including phenazine, siderophore production and terpene were also identified in this study. Our results suggested that these haloarchaeal strains can be used as an eco-friendly biofertilizer to improve growth and productivity in hypersaline environment.

Isolation and Characterization of Plant Growth Promoting Archaea Associated with the Rhizosphere of *Salsola stocksii* and*Atriplex amnicola*

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Abstract

Halophilic microorganisms play a crucial role in plant health and growth in salinity affected lands. The objective of this study was to evaluate the haloarchaeal diversity from the rhizosphere of halophytes and plant growth promoting abilities of these strains. The whole genome sequences of two haloarchaeal strains,*Halorubrum lacusprofundi* HL1RP11 and *Halobacterium noricense* NRS2HaP9, were analyzed, and genes related to plant growth promoting traits were identified. Phylogenetic analysis showed that archaeal strains of *Halococcus, Halorubrum, Halobacterium* and*Natrinema* were dominant in the rhizosphere of halophytes. More than 60% of the strains were positive for phosphate solubilization and IAA production. About 50% of strains were positive for nitrogen fixation, while 33% were siderophore producers. More than 40% of haloarchaeal strains showed the heavy metal resistance for Nickel, Cadmium, Chromium and Zinc at a concentration of 5 mM. Genes involved in plant growth promotion were identified through annotation. Gene clusters related to secondary metabolites including phenazine, siderophore production and terpene were also identified in this study. Our results suggested that these haloarchaeal strains can be used as an eco-friendly biofertilizer to improve growth and productivity in hypersaline environment.

Keywords: Halophilic archaea; plant growth promoting traits; osmoregulation; halophytes; *Halorubrum*

Running Title: Plant Growth Promoting Halophilic Archaea

Introduction

Salinity is one of the most adverse environmental factors limiting the productivity of crop plants. It covers over 6% of the land worldwide [1 3]. Salt stress affects the agricultural land in many ways which include ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on phosphorous uptake from soil [2,4]. It reduces the crop production and has adverse effect on germination due to toxicity and change in enzymatic activities [5,6]. Halophytes are plants that grow well in saline soil and water, such as para grass, *Salsola stocksii, Kochia indica* and*Atriplex amnicola* [7]. Therefore, these offer a better alternative where conventional crops cannot be raised, and drainage is too expensive. They may also contribute significantly to the developing world's supply of food, fiber, fuel and fodder [8].

Halophiles are microorganisms that grow in environments with high salt concentrations. Halophytes rhizosphere harbors a great diversity of halophilic bacterial, archaeal and fungal halophiles. The diverse microbial communities associated with halophyte rhizospheres help these plants to cope with high salinity and drought stress [9,10]. Microorganisms residing in the rhizosphere play a crucial role in plant fitness and productivity, especially under extreme conditions [6,11,12]. Studies of rhizosphere microbiomes have revealed their influence on chemical exudates responsible for the production and secretion of signaling molecules by both microbes and plants [13].

Halophilic archaea grow in environments with salt concentrations varying from 15 to 30% NaCl [14,15]. They can survive at high salt concentrations reaching up to 5M NaCl [16]. Halophilic archaea use 'salt in' for their survival in all high salt concentrations [16,14]. Some halophiles including *Halorhodospira* and*Halorubrum* revealed the use of both strategies for salt adaptation. Previous studies on solar salterns showed the occurrence of a major new phylotype, called nanohaloarchaea with a small cell size of approximately 0.5 μm [17].

Plant growth promoting (PGP) microbes living in the rhizosphere of halophytes play an important role in plant health and soil fertility under salinity stress conditions. They enhance plant growth and increase grain yield of various crops such as wheat, corn, rice, sugarcane and legumes by solubilization of minerals (P, K, Zn), nitrogen fixation, production of compounds such as indole acetic acid (phytohormone), siderophores, HCN, and breaking down of complex organic materials for the easy uptake by plants [18,19]. Only a few studies reported the isolation and characterization of halophilic archaeal genera including*Halobacterium, Natrinema, Haloferax, Natrococcus* and*Haloarcula* from the rhizosphere of halophytes such as*Abutilon, Dicanthium, Sporobolous* and *Sueada* [20 22]. Data on phosphate solubilization, siderophore and IAA (indole acetic acid) production is available from methanogens, haloarchaea and thermococci [23 25]. Halophilic archaeal genera

including*Natrinema, Halobacterium* and *Halococcus* have been reported for P-solubilization and production of organic acids such as citric, succinic, oxalic, lactic, acetic and isovaleric acids [20,26]. Halophilic archaea isolated from hypersaline polluted environments also have the ability to tolerate heavy metal resistance for nickel, cadmium, uranium, chromium and zinc [27-29].

The present work endeavors to identify culturable halophilic archaeal diversity isolated from the rhizosphere of halophytes (*S. stocksii* and *A. amnicola*) and non-rhizosphere soil samples collected from Khewra Salt Mines, Pakistan. This study is the first report on the characterization of plant growth promoting halophilic archaeal strains with their ability to solubilize phosphate, fix atmospheric nitrogen, production of indole acetic acid, siderophores and exopolysaccharides as well as identification of genes and operons involved in plant growth promotion, secondary metabolism, environmental adaptation, glycerol metabolism and membrane transportation from the genomes of *Halorubrum lacusprofundi* HL1RP11 and*Halobacterium noricense* NRS2HaP9.

2. Material and Methods

2.1. Sampling of Soil

Khewra Salt Mine is the world second largest salt mine, located near Pind Dadan Khan Tehsil of Jhelum District, Punjab, Pakistan. Geographically, it is located about 32° 38´ North latitude, 73°10´ East longitude and an elevation of 313-360 meters above the sea level about 200 km from Islamabad. It has Na^+ and Cl^- dominating ions and the pH is near neutral to slightly alkaline. The sampling area was selected according to land use and vegetation cover. Vegetation of this area is classified as sub-tropical dry evergreen forest. Rhizospheric soil samples were collected by gently removing the plants and obtaining the soil attached to the roots. For non-rhizospheric saline soil samples, the upper 8-10 cm of mineral soil was collected. At each site, soil samples of approximately 500 g each from four different locations were collected in black sterile polythene bags. These samples were stored at 4 for further analysis.

2.2. Isolation of Halophilic Archaea from the Rhizospheric and Non-rhizospheric Soils of *Salsola stocksii* and *Atriplex amnicola*

Modified growth medium (MGM) with NaCl 250 g/L, casamino acids 7.5 g/L, peptone 5 g/L, yeast extract 2 g/L, KCl 30 g/L, trisodium citrate 3 g/L, $MgSO_4$ 24 g/L, pH 7.2; was used for the isolation and purification of archaea from rhizospheric and non-rhizospheric soils of*Salsola stocksii* and *Atriplex amnicola* [30].These soil samples were collected from Khewra Salt Mine, Pakistan. For isolation of individual archaeal strains, the soil sample was mixed thoroughly, sieved and then one gram of the representative soil sample was used for further analysis. All samples were serially diluted upto 10^{-10} and 100 µL of each dilution from 10^{-3} to 10^{-6} were spread on MGM plates for counting colony forming units (CFU) per gram of soil (dry weight). Plates were incubated at 37 °C until the appearance of archaeal colonies. Single, well-isolated colonies were selected, grown in MGM broth overnight, and stored in 33% glycerol at -80 °C for subsequent characterization.

2.3. Isolation of Genomic DNA and Amplification of 16S rRNA Gene

Genomic DNA of halophilic archaeal strains from the rhizospheric and non-rhizospheric soils was isolated by CTAB method [31]. The quality and quantity of extracted DNA was observed by agarose gel electrophoresis in TAE buffer (Tris base, 242 g/L; glacial acetic acid, 57 ml/L; 0.5 M EDTA, 100 ml/L) and the Nanodrop, respectively.

For PCR amplification of 16S rRNA gene, universal forward primer HA1 (5'-ATTCCGGTTGA

TCCTGCCGGAGGTC-3'), and reverse primer HA1465 (5'-GATCCAGCCGCAGATTCCCC-3'), for prokaryotes were used [32]. Denaturation temperature was 95 for 5 min followed by 35 rounds of 94 for 60 sec, 55 for 50 sec and 72 for 90 sec and final extension at 72 for 10 min. A reaction mixture of 25 µL was prepared by using Taq buffer 2.5 µL (10X), $MgCl_2$ 3 µL (25 mM), Taq polymerase 1 µL, dNTPs 2 µL (2.5 mM), 2 µL of forward and reverse primers (10 pmol) and the template DNA 2 µL (>50ng/ µL). PCR products were purified by using gel extraction kit (Fermantas USA). Agarose gel (1%) method and NanoDrop equipment

were used to determine the quality and quantity of the sample, respectively. Purified PCR products were sequenced by using forward and reverse primers (Eurofins, Germany).

2.4. Sequencing and Phylogenetic Analysis

Amplified gene of 16S rRNA was sequenced to identify the archaeal genera and species. The sequences were submitted to NCBI. Based on results obtained by BLAST sequence, A phylogenetic tree was constructed by neighbor-joining method with the help of software MEGA 10 [33]. Test of phylogeny was done by using bootstrap method (1000 replicates). The 16S rRNA sequences of bacterial strains were deposited in the GenBank with accession numbers LT634693- LT634709.

2.5. Screening of Archaeal Strains for Salt, pH and Temperature Tolerance Ability

Archaeal strains were grown in the presence of different salt concentrations (2.0-5.0M NaCl), pH ranges 4-12 and temperature range 4-56 by using MGM broth medium. Strains were cultured in 250 ml flasks with continuous rotatory agitation at 150 rpm for 72 h. During incubation, archaeal growth was measured at different time intervals (3, 6, 12, 24, 48 and 72 h) by observing their OD at 600 nm.

2.6. Assays for Plant Growth Promotion

2.6.1. Phosphate Solubilization Assay

Pikovskaya phosphate medium (PVK) with the following composition (g/L): 10.0 glucose, 1.0 yeast extract, 5.0 tri-calcium phosphate (TCP), 200.0 NaCl, 5.0 KCl, 31 $MgCl_2.6H_2O$, 35.0 $MgSO_4.7H_2O$, 0.5 $CaCl_2.2H_2O$ and 0.5 KH_2PO_4 (pH 7.2) was used to test the phosphate solubilizing ability of haloarchaea [34]. For inoculation onto plates, the archaeal strains were grown in a liquid medium until stationary phase at which time the cells were harvested by centrifugation (8000xg, 10 min) and the pellets washed with sterile water (SW) three times to remove any traces of the medium. The cell pellets were then diluted to OD600 = 0.2 in SW. Ten µL droplets were spotted onto the plates and allowed to air dry. then incubated upside down at 37 for 5 days and the size of the clearing zone around the colony was measured as an indication of positive activity.

Quantitative analysis of P-solubilization of archaeal strains was done by molybdate blue color method [35]. Available phosphate was calculated after 7 and 14 days. Cell-free supernatants were used for the quantification of P-solubilization. After recording pH of cell-free supernatants, they were filtered through 0.2 µm sterile filters (Orange Scientific GyroDisc CA-PC, Belgium) to remove any residues. Solubilized phosphates (primary and secondary orthophosphate) were measured by spectrophotometer (Camspec M350-Double Beam UV-Visible Spectrophotometer, UK) at 882 nm and values were calculated by using a standard curve (KH_2PO_4 using 2, 4, 6, 8, 10, 12 ppm solutions).

2.6.2. Indole Acetic Acid (IAA) Production Assay

For assessment of IAA, archaeal cultures were inoculated in 100 mL sterile MGM broth containing 0.1% L-tryptophan and the flasks were kept in shaking incubator at 120 rpm, 37 for 7 days. The culture medium was transferred to sterile falcon tubes and centrifuged at 10,000 rpm for 15-20 minutes. The supernatant was transferred to a flask and pH was adjusted at 2.8 using hydrochloric acid. Later, it was extracted twice with equal volumes of ethyl acetate. The clear solution was transferred to a beaker and anhydrous sodium sulfate was added to absorb left over moisture. The clear solution was evaporated until dry using rotary evaporator and was resuspended in 1 mL methanol. These samples were analyzed by high-performance liquid chromatography (HPLC; Waters; e2995, separations module) with 299h photodiode-array (PDA) detector using a Nucleosil C18 column (4.6×250 mm, 3µM; Macherey-Nagel, Germany). The mobile phase was a mixture of methanol/acetic acid/water (30:1:70, v/v/v) and the flow rate was adjusted at 1.2 mL/min. Pure indole-3- acetic acid (Sigma) was used to prepare standard solutions and IAA production was quantified.

2.6.3. Acetylene Reduction Assay

Nitrogenase activity was assessed by using acetylene reduction assay. Single archaeal colonies of each strain

were inoculated in vials containing modified NFM (nitrogen free malate) semi-solid medium (5 mL/vial) and incubated at 37 . NFM semi-solid medium contained (g L-1) malic acid, 5.0; K2HPO4, 0.6; KH2PO4, 1.8; MgSO4.7H2O, 0.2; NaCl, 0.1; CaCl2.2H2O, 0.02; micronutrient solution (above), 2 mL; bromothymol blue (5 g L-1 in 0.2 N KOH), 2 mL; FeEDTA (16.4 g L-1), 4 mL; vitamin solution, 1mL and NaCl, 200 g L-1; adjusted pH to 6.8 with KOH and 2% agar [36]. After 48 h of growth, acetylene (10% v/v) was injected, and vials were re-incubated for 24 h. Ethylene production was measured by GC-2014 System (Shimadzu Corporation, Japan) fitted with a Porapak column using flame ionization detector. Helium was used as a carrier gas. Nitrogenase activity was described in terms of nanomoles of ethylene per hour per milligram archaeal protein [37].

2.6.4. Siderophore Productions Assay

CAS (chrome azurol S) agar medium devoid of nutrients was used as an indicator of siderophore production. The components needed for a liter of the overlay medium were: 0.5 M MOPS buffer, 10 g $MgSO_4.7H_2O$, 1 g $CaCl_2.2H_2O$, 50 mL of Solution I (CAS) (0.065 g in 50 mL H_2O), 10 mL of Solution II (0.135 g $FeCl_3.2H_2O$ in 500 mL H_2O), 40 mL of Solution III (0.0729 g CTAB in 40 mL H_2O) and 9 g Bacto-Agar [38]. Ten mL of the overlay medium was spread on culture plates of selected strains grown at 37 for 4 days on solid MGM medium. After a maximum period of 15 min, a color change in the blue medium was considered positive for siderophore production. The experiment was repeated three times.

2.7. DNA Isolation, Genome Sequencing and Assembly of*Halorubrum lacusprofundi* HL1RP11 and *Halobacterium noricense* NRS2HaP9

Based on the results of plant growth promoting traits, two archaeal strains *Halorubrum lacusprofundi* HL1RP11 and *Halobacterium noricense* NRS2HaP9 were selected for whole genome sequence analysis and identification of PGP-related genes. These strains were grown into 100 mL MGM broth overnight at 37 °C with 125 rpm. The Genomic DNA was isolated using Genomic DNA isolation kit (Thermo Scientific GeneJET, USA). Genome sequencing of these haloarchaeal strains was performed with the Illumina HiSeq2000® sequencing platform. For the current study, 5 µg of genomic DNA was extracted from each archaeal strain and was prepared for genome sequencing using the Illumina HiSeq2000® library preparation kit (Illumina, Inc.), following the manufacturer's instructions. The sequencing data was then assembled into complete contigs with SPAdes assembler Version 3.13.0 [39]. The contigs were arranged against the genomes of *Halorubrum lacusprofundi* ATCC 49239 and*Halobacterium noricense* A1 by using Mauve [40].

2.8. Genome Annotation of *Halorubrum lacusprofundi*HL1RP11 and *Halobacterium noricense* NRS2HaP9

The de novo gene prediction was performed by using GeneMarks and CLC genomics workbench [41]. The functional classification was conducted through COG (corresponding cluster of orthologous groups of protein) analysis. The gene function was annotated by BLAST against Kyoto Encyclopedia of Genes and Genomes database KEGG pathway [42]. KEGG Orthology Based Annotation System (KOBAS 2.0) was used for functional analysis of genes. To predict genes and operons involved in secondary metabolism and antibiotic resistance antiSMASH 4.0 software was used [43]. The whole genome sequences of strains HL1RP11 and NRS2HaP9 were deposited in the GenBank database under the accession number JAJNEG000000000 and JAJSOI000000000.

Results

3.1. Isolation and Identification of Halophilic Archaeal Strains

A total of 24 halophilic archaeal isolates were characterized and identified from the rhizospheric and non-rhizospheric soil samples of*S. stocksii* and *A. amnicola* (**Table 1; Fig. 1**). These isolates were identified based on 16S rRNA gene analysis. Four strains showed more than 99% homology with*Halobacterium* spp., four strains were related to*Halomicrobium* spp., three strains were identified as*Halococcus* spp., three strains were related to *Natrinema*spp., two strains NRS3HaP17 and LK4HAP18 were belonging to*Natrialba* spp.,

one strain HL1RS17 was identified as*Halolamina sediminis* , one strain AT3RS21 was identified as*Haloferax denitrificans* and one strain AT4RS18 was identified as*Halalkalicoccus jeotgali* (**Table 1; Fig. 1**).

Phenotypic Characterization of Haloarchaea

The halophilic archaeal cultures were grown on MGM agar. On incubation, translucent and opaque colonies with orange, red or pink pigmentation were observed. As shown in **Fig. 2A** , haloarchaeal strains were extremely halophiles and only few strains were able to grow under 2M of total salt but grew optimally at 2.5 - 4.0M of salt (NaCl). These strains were able to grow at a wide range of temperature with 37-42 °C optimum temperature (**Fig. 2B**). Mostly strains grew well at pH range from 6 to 8, however, some strains did grow at 55 °C and the pH of 9.5 (**Fig. 2C**).

Plant Growth Promoting Potential of Halophilic Archaea

Halophilic archaeal strains were screened for various plant growth promoting (PGP) abilities such as IAA production, P-solubilization, nitrogen fixation and siderophore production. Most of the strains showed more than two PGP abilities (**Table 2; Fig. S1**). Twenty-one strains showed P-solubilization activity with a range from 8.15 to 87.77 µg/mL. *Natrinema gari* strain HL1RP1 had the maximum P-solubilization activity (87.77 µg/mL). Fifteen strains showed production of IAA with a range from 2.11 to 63.42 µg/mL. Only 12 strains showed nitrogen fixation ability and 8 strains showed positive results for siderophore production assay (**Table 2; Fig. S1**).

Heavy metal resistance profile of bacterial strain

More than 90% of the rhizospheric archaeal strains showed heavy metal tolerance for Cd, Ni, Cr and Zn at a concentration of 1 mM, 71-85% archaeal strains showed heavy metal tolerance at a concentration of 2.5 mM, 40-63% archaeal strains showed heavy metal tolerance at a concentration of 5.0 mM, and only a few archaea strains (0-19%) showed tolerance at a concentration of 10 mM (**Fig. 3**).

3.5. Features of Genomes of*Halorubrum lacusprofundi* HL1RP11 and *Halobacterium noricense* NRS2-HaP9

The size of genomes of *Halorubrum* HL1RP11 and*Halobacterium* NRS2-HaP9 was 3,550,491 and 3,037,489 bp, respectively (**Fig. S1**). In the genome of *Halorubrum*HL1RP11 strain, a total of 3517 genes were predicted with 3,431 protein coding sequences (CDs) and RNA related genes were 55 (**Table S1**). In the genome of *Halobacterium* NRS2-HaP9 strain, a total of 3228 genes were predicted with 3155 protein coding sequences (CDs) and RNA related genes were 51 (**Table S1**). Plasmid sequence was not identified in both strains.

Functional Annotation of *Halorubrum lacusprofundi*HL1RP11 and *Halobacterium noricense* NRS2-HaP9 Genomes

Most of the unique genes were predicted to code hypothetical proteins in both genomes. In the genome of *Halorubrum lacusprofundi* HL1RP11, out of 3,431 proteins, 1354 (49.7%) were assigned to COG functional categories while in case of *Halobacterium noricense* NRS2-HaP9 genome, out of 3155 proteins, 1419 (44%) were assigned to COG functional categories (**Fig. 4**). The functional analysis of these genes using KEGG pathway database showed that they have an important role in various metabolic pathways including plant growth promotion, environmental adaptation, bioremediation of different toxic compounds, heavy metals, and other abiotic stresses. The functional analysis of CDSs showed that they could be classified into 19 general COG categories including the metabolism of carbohydrates, amino acids, lipids, transcription, energy, cofactors and vitamins, inorganic ions, signal transduction and cellular processes, glycan biosynthesis and metabolism, nucleotide metabolism, secondary metabolites, Iron acquisition and metabolism and xenobiotics biodegradation (**Fig. 4**).

Prediction of Genes Related to Plant Growth Promotion

The functional analysis of *Halorubrum* HL1RP11 and NRS2HaP9 genomes identified different genes involved

in plant growth promotion of halophytes. The presence of genes involved in tryptophan biosynthesis (indole acetic acid production), and tyrosine such as *trpA, trpB, trpC, trpD, trpE, trpG, tyrA2* and *pheA2* was confirmed by genome analysis of both HL1RP11 and NRS2HaP9 strains. Eleven genes including*afuB, fbpB, afuA, fbpA, menF, ABC.FEV.S, ABC.FEV.P, ABC.FEV.A, feoA, FRD* and *adhB-1* involved in iron metabolism and siderophore production were identified in the genomes of both haloarchaeal strains (**Table S2; Fig. 5, 6A and 6B**). Presence of genes including*PstA, PstB, gcd, pqq_1, PiT* and *phoU* related to P-solubilization (PQQ-dependent alcohol dehydrogenase) were predicted in HL1RP11 and NRS2HaP9 genomes. Genes related to nitrogen fixation, nitrogenase protective and regulatory proteins such as *nifU, nif3, nifU_N, glnK2* and *glnB* were also detected in genomes of both HL1RP11 and NRS2HaP9 strains. Putative NAD(P)H nitroreductase gene*ydfN* was detected in genome of NRS2HaP9 strain (**Table S2; Fig. 5, 6A and 6B**). Few genes including *pgdA, dgoD, EOI, rhaM* and *uxuA* related to production of exopolysaccharides were also predicted in this study (**Table S2; Fig. 5, 6A and 6B**). When PGP genes from the genomes of HL1RP11 and NRS2HaP9 were compared with their reference strains, there was a difference in the number of genes especially siderophore production, nitrogen fixation and phosphate solubilization related genes (**Fig. 6A and 6B**).

Prediction of Genes Related to Environmental Adaptation, Glycerol Metabolism and Membrane Transport

Genes potentially involved in environmental adaptation, glycerol metabolism and membrane transport have been identified in the genomes of halophilic archaea. Genes for environmental adaptation (*ACSL, fadD, COX10, ctaB, cyoE, COX15, ctaA, RP-S6e, RPS6* and *psd*), glycerol metabolism (*pssA, gldA, ALDH, dhaK, dhal, dhaM, SQD1, sqdB, gck, gckA, dgs, bgsA, mgs, bgsB, araM, egsA, glpA, glpD, glpB, glpC, pgsA, PGS1* and *carS*) and 63 genes related to amino acids, sugar molecules, iron, phosphate, thiamine, biotin, zinc, sulfonate and arabinogalactan transportation and signal recognition proteins were identified in the genome of *Halorubrum lacusprofundi* HL1RP11 (**Tables S3 & S4**). Based on functional analysis of*Halobacterium noricense* NRS2-HaP9 genome, genes for environmental adaptation (*ACSL, fadD, COX10, ctaB, cyoE, COX15, ctaA, RP-S6e, RPS6* and *psd*), glycerol metabolism (*pssA, gldA, ALDH, gck, gckA, dgs, bgsA, bgsB, araM, egsA, glpA, glpD, glpB, glpC,* and*carS*) and 44 genes related to amino acids, sugar molecules, iron, phosphate, thiamine, biotin, copper, molybdate, sulfonate, nucleotides and urea transportation and some signal recognition proteins were identified (**Tables S3 and S4**). When genomes of HL1RP11 and NRS2HaP9 were compared with their reference strains, there was a difference in the number of genes especially related to environmental adaptation, and membrane transport (**Fig. 6C and 6D**).

Prediction of Genes Related to Heavy Metal Resistance

Based on functional analysis of *Halorubrum lacusprofundi* HL1RP11, 159 genes related to heavy metal resistance including Nickel, Cadmium, Antimony, Arsenic, Iron, Chromium and Zinc

were identified (**Tables S5; Fig. 7**) while in the genome of*Halobacterium noricense* NRS2-HaP9, 171 genes related to heavy metal resistance were identified (**Tables S5; Fig. 7**). When genomes of HL1RP11 and NRS2HaP9 were compared with their reference strains, we observed a clear difference in number of genes related to heavy metal resistance (**Fig. 7**).

Production of Secondary Metabolites

In the genome of *Halorubrum lacusprofundi* HL1RP11, antimicrobial gene clusters including thiopeptides (thiazolyl peptides), bacteriocins and terpenes were identified (**Fig. S3**). Secondary metabolites encoding gene clusters such as siderophore, thiopeptides and terpenes were identified in the genome of *Halobacterium noricense*NRS2-HaP9 (**Fig. S3**). These genes might be involved in plant growth improvement and biocontrol mechanisms.

Discussion

Rhizosphere microbial communities from extreme environments such as arid, saline, alkaline and acidic are more complex than soils having a neutral pH and exhibiting moderate salinity. The rich microbial diversity of

halophyte rhizospheres helps these plants cope with high salinity and drought [6]. In the current study, culturable halophilic archaeal diversity was analyzed from the rhizosphere and non-rhizospheric soils of halophytes including *S. stocksii* and*A. Amnicola* . Plant growth promoting traits of the isolated archaeal strains were screened using different selective media and genes related to plant growth promotion, secondary metabolism and osmoregulation were identified through whole genome sequence analysis of*Halorubrum* HL1RP11 and *Halobacterium* NRS2-HaP9 strains.

Based on 16S rRNA gene analysis, a total of 24 halophilic archaeal isolates were identified from the rhizospheric and non-rhizospheric soil samples of *S. stocksii* and *A. amnicola.* In this study, 9 halophilic genera including *Halobacterium, Halomicrobium, Halorubrum, Halococcus, Haloferax* and *Halalkalicoccus* were identified. *Halobacterium, Halomicrobium, Halococcus* and*Natrinema* were more abundant as compared to other genera from the rhizosphere of halophytes. A number of previous studies have reported the halophilic archaeal genera such as *Halobacterium,Halococcus, Halomicrobium, Haloferax* and *Halalkalicoccus*from various aquatic hypersaline environments [44-47] but only two studies including Yadav et al. [20] and Dubey et al. [48] reported the PGP halophilic archaea from the rhizosphere of halophytes. Haloarchaeal strains isolated in this study were extremely halophiles and a few strains were able to grow under 2M of total salt but grew optimally at 2.5-4.0M of salt (NaCl).

Halophilic archaeal strains were orange to red in color because of red pigmented protein bacterioruberin. Haloarchaeal enzymes usually use KCl ions to work at high salinity levels [4, 14,15]. Haloarchaeal strains from the genera *Halobacterium* and *Halococcus* can synthesize L-glutamate as osmolyte. They have intracellular gas vesicles which are usually filled with different gases and provide buoyance and enable cells to regulate their position in the aquatic environments [15, 49]. *Halobacterium salinarum* contains fibrillary structures (fibrocrystalline bodies) which represent the presence of cytoskeleton-like organelle in haloarchaeal cells [50].

In this study, most of the strains showed more than two PGP abilities. About 87.5% of haloarchaeal strains showed P-solubilization activity with a range from 8.15 to 87.77 µg/mL, 62.5% of strains showed production of IAA with a range from 2.11 to 63.42 µg/mL, 50% of strains showed nitrogen fixation ability, and 33% strains showed positive results for siderophore production assay. From the rhizosphere of halophytes, halophilic archaea such as *Natrialba, Natrinema, Haloarcula,* and *Halococcus* with the ability to solubilize phosphate, produce IAA, and siderophores have been previously reported. These archaeal strains showed mineral phosphate solubilization by the production of organic acids such as oxalic, acetic, citric, and succinic acid [20, 51].

A recent study also showed that haloarchaea have the ability to produce indole acetic acid and other phytohormones [52]. Some ammonia-oxidizing archaea, e.g., *Nitrosopumilus maritimus* and halophilic archaea, e.g., *Halobacterium salinarum* and*Haloferax volcanii* are capable of siderophore production. Archaea use iron acquisition and produce siderophores [53-55]. Some archaeal groups play an important role in nitrogen cycle. A number of previous studies have reported the ammonia oxidizing methanogens [24, 56].

The whole genome analysis of *Halorubrum* HL1RP11 and*Halobacterium* NRS2-HaP9 revealed that there were 3431 and 3155 protein coding sequences respectively. A large number of proteins were annotated as hypothetical proteins. The functional analysis of*Halorubrum* HL1RP11 and *Halobacterium* NRS2-HaP9 genomes using KEGG pathway database showed that it has an important role in metabolism of carbohydrates, amino acids, lipids, energy, cofactors and vitamins, inorganic ions, glycan biosynthesis and metabolism, secondary metabolites, signal transduction and cellular processes, DNA replication and repair, cell motility, transcription, translation, ribosomal biogenesis, abiotic stresses and bioremediation of different toxic compounds. Some recent studies on genome sequence analysis of haloarchaea revealed that these microorganisms have a large diversity of proteins and enzymes involved in different metabolic pathways, abiotic stress management and plant growth promotion [57-59].

The functional analysis of *Halorubrum* HL1RP11 and*Halobacterium* NRS2HaP9 genomes revealed the identification of various genes involved in plant growth promotion. The presence of genes including *PstA, PstB, gcd, pqq_1, PiT* and *phoU* related to P-solubilization (PQQ-dependent alcohol dehydrogenase) were pre-

dicted in this study. Genes involved in tryptophan biosynthesis (indole acetic acid production), and tyrosine were also identified by genome analysis of both HL1RP11 and NRS2HaP9 strains. Eleven genes involved in iron metabolism and siderophore production were identified in the genomes of both haloarchaeal strains. Genes related to nitrogen fixation, nitrogenase and related regulatory proteins (*nif3, nifU, nifU_N, glnB* and *glnK2*) were also detected in genomes of both HL1RP11 and NRS2HaP9 strains.

Some previous studies also reported the role of archaea in plant growth promotion with their ability to solubilize inorganic phosphate and produce phytohormones and siderophores [20, 60,61]. The ammonia monooxygenase genes were identified in ammonia-oxidizing archaea that were isolated from the rhizosphere of *Littorella uniflora*[24]. This study is the first report of nitrogen-fixing halophilic archaea based on biochemical detection as well as identification of genes related to nitrogen fixation by using whole genome analysis.

Halophilic archaeal genera including *Haloarcula, Halococcus, Haloferax, Halobacterium* and *Natronococcus* have the ability to produce exopolysaccharides. Various sugars such as glucose, rhamnose, galactose, mannose, galactosamine, and glucopyranosiduronic acid are involved in the biosynthesis of exopolysaccharides in archaea [62, 63]. Identification of genes related to PGP traits has been previously reported in some archaea, however, this study is the first report that described the characterization of PGP haloarchaeal strains from the rhizosphere of halophytes and identification of related genes through whole genome analysis of two haloarchaeal strains *Halorubrum*HL1RP11 and *Halobacterium* NRS2HaP9.

About 40-63% of archaeal strains showed heavy metal tolerance for Cd, Ni, Cr and Zn at a concentration of 5.0 mM. In this study, haloarchaea showed more tolerance for chromium as compared to other metals. A number of previous studies also showed that halophilic archaea including*Haloferax* , Halobacterium, *Halococcus* , *Haloarcula* , and *Halorubrum* have heavy metal resistance genes on their plasmids and chromosomal DNA. These microorganisms help plants to grow under saline polluted soils [27-29].

Genome annotation analysis showed that siderophore, thiopeptides and terpenes were commonly identified from both *Halorubrum* HL1RP11 and *Halobacterium* NRS2-HaP9. Thiopeptides (thiazolyl peptides) are a class of pretentious antibiotics produced by bacteria and archaea. These antibiotics usually show positive activity against Gram-positive bacteria such as *Bacillus* and *Staphylococcus* genera [59, 64]. Terpenes and terpenoids are important antimicrobial compounds identified and characterized in bacteria, archaea, and plants. Terpenes and terpenoids play a role in biosynthesis of the cell membrane and cell wall, electron transport and conversion of light into chemical energy including chlorophylls, bacteriochlorophylls, rhodopsins, and carotenoids [65, 66]. Some recent studies reported the identification of gene clusters related to secondary metabolism in archaea [67], however, this study is the report of identification of gene clusters related to secondary metabolism in halophilic archaea.

In summary, our results suggest that *Halobacterium, Halococcus, Halorubrum, Halobacterium* and *Natrinema* were dominant in all soils. Most of the strains identified in this study showed more than two PGP traits. More than 60% strains demonstrated positive results for P-solubilization, IAA production and heavy metal resistance. The genomic annotation of *Halorubrum* HL1RP11 and *Halobacterium*NRS2HaP9 revealed the identification of genes involved in PGP, e.g., phosphate solubilization, IAA production, nitrogen fixation, and exopolysaccharides production; heavy metal resistance and secondary metabolism, e.g., phenazine, siderophore production and terpene related gene cluster. We suggest that PGP haloarchaeal strains may be used as an eco-friendly biofertilizer that will be a better alternative to chemical fertilizers for improving plant growth under salinity affected agricultural lands.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.

nih.gov/, LT634693- LT634709 and JAJNEG000000000 and JAJSOI000000000.

Author contributions

SMu: conducted the experiment, analyzed the data, and prepared the manuscript. HZS conducted the experiment and helped in data analysis. SMe: guided in experiment plan and edited the manuscript. KM: supervised the research and edited the manuscript. All authors contributed to the article and approved the submitted version.

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