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Identification of plasmid encoded osmoregulatory genes from halophilic bacteria isolated from the rhizosphere of halophytes

Salma Mukhtar[a,b], Samia Ahmad[c], Aftab Bashir[a], Samina Mehnaz[a], Muhammad Sajjad Mirza[d], Kauser Abdulla Malik[a,*]

[a] School of Life Sciences, Forman Christian College (A Chartered University), Ferozepur Road, Lahore 54600, Pakistan
[b] School of Biological Sciences, University of the Punjab, Lahore, Pakistan
[c] Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI 48824, USA
[d] Environmental Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Jhang Raod, Faisalabad, Pakistan

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ABSTRACT

Bacterial plasmids carry genes that code for additional traits such as osmoregulation, CO_2 fixation, antibiotic and heavy metal resistance, root nodulation and nitrogen fixation. The main objective of the current study was to identify plasmid-conferring osmoregulatory genes in bacteria isolated from rhizospheric and non-rhizospheric soils of halophytes (*Salsola stocksii* and *Atriplex amnicola*). More than 55% of halophilic bacteria from the rhizosphere and 70% from non-rhizospheric soils were able to grow at 3 M salt concentrations. All the strains showed optimum growth at 1.5–3.0 M NaCl. Bacterial strains from the *Salsola* rhizosphere showed maximum (31%) plasmid elimination during curing experiments as compared to bacterial strains from the *Atriplex* rhizosphere and non-rhizospheric soils. Two plasmid cured strains *Bacillus* HL2HP6 and *Oceanobacillus* HL2RP7 lost their ability to grow in halophilic medium, but they grew well on LB medium. The plasmid cured strains also showed a change in sensitivity to specific antibiotics. These plasmids were isolated and transformed into *E. coli* strains and growth response of wild-type and transformed *E. coli* strains was compared at 1.5–4 M NaCl concentrations. Chromosomal DNA and plasmids from *Bacillus filamentosus* HL2HP6 were sequenced by using high throughput sequencing approach. Results of functional analysis of plasmid sequences showed different proteins and enzymes involved in osmoregulation of bacteria, such as trehalose, ectoine synthetase, porins, proline, alanine, inorganic ion transporters, dehydrogenases and peptidases. Our results suggested that plasmid conferring osmoregulatory genes play a vital role to maintain internal osmotic balance of bacterial cells and these genes can be used to develop salt tolerant transgenic crops.

1. Introduction

Water is one of the most important prime elements responsible for life. Halophiles have adapted special genetic and physiological modifications to survive in extreme (hypersaline) environments (Irshad et al., 2014; Youssef et al., 2014). They can be classified as slightly, moderately or extremely halophilic depending on their requirement for sodium chloride (DasSarma and DasSarma, 2015; Purdy et al., 2004). Halophilic microorganisms generally use two mechanisms of adaptation to hypersaline environments: (1) the accumulation of compatible solutes (osmolytes) to the imposed osmotic pressure and (2) the maintenance of high intracellular ionic (K^+) concentrations and adaptation of entire intracellular enzymatic machinery to function in hypersaline conditions. Mostly halophilic bacteria and archaea have two component regulatory systems. They have osmosensor and osmoregulator proteins to recognize osmotic imbalance. Regulator proteins bind DNA and regulate gene expression and sensor proteins (histidine kinases) detect the signals. This system controls the changes in osmotic pressure on both sides of the cell membrane (Foo et al., 2015).

Mostly halophilic bacteria balance their cytoplasm with the high salt concentration of the medium by the accumulation of osmolytes. These compatible solutes are excreted in the cytoplasm either by the bacterial cell itself or can be taken from the medium. Osmolytes can be classified into two main groups; organic solutes (trehalose, ectoine, betaine, proline), inorganic solutes (K^+, Mg^{2+}, Na^+) (Nath, 2016). Mostly bacteria lack intracellular systems for active transport of water to cope with external osmotic stress. Therefore, they balance internal environment by the transport or synthesis of a group of organic solutes

* Corresponding author.
E-mail address: kausermalik@fccollege.edu.pk (K.A. Malik).

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(compatible solutes) without affecting the metabolic function of the cell (Moghaddam et al., 2016; Petrovic et al., 2004). Compatible solutes can be classified according to their chemical nature; (1) anionic solutes, (2) zwitterionic solutes and (3) non-charged solutes (Culla et al., 1999). 'Salt in' strategy is used by two phylogenetically unrelated groups; anaerobic halophilic bacteria (Haloanaerobiales) and aerobic halophilic archaea (Halobacteriales). These groups use inorganic ions (K^+, Mg^{2+}, Na^+) to cope with external osmotic stress (high salt concentrations). All intracellular machinery is adapted to the high salt concentration in the external environment of the cell (Karan et al., 2012).

Mostly halophilic bacteria contain several plasmids. Plasmid conferring genes in bacteria play an important role in bacterial flexibility and adaptation to different abiotic stresses (Dziewit et al., 2015; Frage et al., 2016). Bacterial plasmids carry genes that codes for additional traits such as osmoregulation (Klumper et al., 2015; Oren, 2012), induction of plant tumors, CO_2 fixation, antibiotic and heavy metal resistance (Rahube et al., 2014), root nodulation and nitrogen fixation, different metabolic transformation, virulence and plant tumor induction (Norberg et al., 2011b). Microbial plasmids also determine some specific phenotypic characteristics of bacteria, such as colony colour (pigments) and mucoid colonial growth (Delmont et al., 2015; Garcillan-Barcia et al., 2011).

Khewra Salt Mine, Pakistan provides a rich and extensive habitat for halophytes and halophilic microorganisms. *Atriplex* and *Salsola* are important biomass producers in the barren lands of this area (Dagla and Shekhawat, 2005). Rhizosphere microbiome of halophytes contains of a wide diversity of halotolerant and halophilic microorganisms (Mukhtar et al., 2017). The main objective of this research was to identify plasmid-conferring osmoregulatory genes in bacteria isolated from the rhizospheric and non-rhizospheric soils of *Salsola stocksii* and *Atriplex amnicola*. Plasmid mediated salt tolerance was studied by using plasmid curing of halophilic strains and then plasmids from halophilic bacterial strains were transformed into *E. coli* strains (Top10 and DH10α). We also compared the antibiotic resistance profile of wild-type and plasmid cured halophilic strains with the transformed *E. coli* strains with plasmids from halophilic bacteria. The current study is the first report of its kind that it deals with the identification of osmoregulatory genes from the rhizospheric halophilic *Bacillus* strain HL2HP6 through whole genome sequence analysis.

2. Materials and methods

2.1. Screening of halophilic bacterial strains with respect to their tolerance ability

Forty-six halophilic bacterial strains from the rhizosphere of *Salsola stocksii,* 42 strains from the rhizosphere of *Atriplex amnicola* and 24 strains from non-rhizospheric soils of halophytes were selected from our lab collection (Table S1). These strains were screened for their salt tolerance by using HaP (Halophilic) medium (Tryptone 5 g/l, Yeast Extract 1 g/l, 5 g/l KCl, 10 g/l $MgSO_4$, 2 g/l K_2HPO_4 and pH 7.2) supplemented with 1.5–4 M NaCl concentrations. Bacterial strains were cultured in 250 mL flasks at 37 °C with continuous rotatory agitation at 130 rpm for 72 h. During incubation, bacterial growth in terms of optical density (OD 600) was measured after different time intervals. Readings were taken after 3, 6, 12, 24, 36, 48 and 72 h of incubation (Mukhtar et al., 2019).

2.2. Plasmid curing halophilic bacterial strains

Halophilic bacterial strains were grown by using HaP medium supplemented with acridine orange (0.10 mg/ml). Sub-culturing of the strains was done again in HaP medium with 2 M NaCl (control) as well as in LB medium and incubated overnight at 37 °C with at 130 rpm. The above procedure was repeated for 3–4 times until no growth was formed in HaP medium (control) but the growth was observed only in LB broth. From LB medium, serial dilutions were made and plated on HaP medium to check sensitivity to NaCl (Akinjogunla and Enabulele, 2010; Durve et al., 2013).

2.3. Isolation and restriction analysis of plasmid DNA from halophilic strains

Plasmid DNA was isolated from halophilic bacterial strains by using QIAGEN® Plasmid MaxiPrep and Purification kit. The concentration of plasmid DNA was measured on 0.8% agarose gel and quantified using the Nanodrop (NanoDrop 200c Thermo Scientific, USA). Plasmid DNA was digested with *Eco*RI and *Bam*HI and incubated at 37 °C in a dry bath for 3 h. The digested DNA was separated on 1% agarose gel along with 1 kb DNA ladder (Fermentas) for 7 h at 30 V.

2.4. Transformation of plasmids from halophilic bacteria into E. coli

Plasmids from halophilic bacterial strains were transformed into *E. coli* strains (Top10 and DH10α) by electroporation and conjugation methods while no DNA was added to these cells in the control sets (Qureshi and Malik, 1990). To check the competency of *E. coli* cells, transformed cells were grown on HaP medium with 1.5 M NaCl concentration. The positive transformants of *E. coli* were screened for salt tolerance by using different NaCl concentrations (1.5–4 M NaCl).

2.5. Antibiotic resistance profile of parental and plasmid cured halophilic strains

The antibiotic resistance pattern of bacterial strains was studied according to Kirby-Bauer disk diffusion method (Bauer et al., 1966; El-Sayed and Helal, 2016). Seven antibiotics, ampicillin (AMP), tobramycin (TOB), amoxicillin (AM), erythromycin (E), sulfamethoxazol/trimithoprim (SXT), penicillin (P) and vancomycin (VA) were used to check antibiotic sensitivity of wild-type and cured halophilic strains and transformed *E coli* strains. Antibiotic discs were placed over freshly prepared HaP medium seeded with bacterial strains under study. All antibiotic disks were placed on each of the seeded plates at appropriate distances from one another and plates were incubated at 37 °C for 24 h. the strains were classified as sensitive or resistant by the presence or absence of inhibition zone of growth around antibiotic discs.

2.6. Sequencing of plasmids from Bacillus filamentosusHL2HP6

Bacillus filamentosus HL2HP6 lost its complete salt tolerance after plasmid curing, so we selected this strain for whole genome sequencing and for identification of plasmid encoded salt tolerant proteins and enzymes. Plasmids from this strain were sequenced by using whole genome sequencing technique. SMART bell sequencing library was constructed using 500–1000 ng size-selected DNA with Pacific Biosciences DNA template Pre Kit 2.0. The binding of SMART bell templates to polymerases was carried out using the Binding Kit P5 and V2 primers. Sequencing was carried out on the Pacific Biosciences RSII platform using C3 reagents. The files resulting from the PacBio sequencing were used directly for the assembly process. Raw reads were processed into sub-reads by removing adaptors and filtered using SMART Analysis 2.2. The filtered sub-reads were used in the HGAP assembly process. An in-house lengths that would give a coverage around 10. For *Bacillus* sp. HL2HP6, seed length 5 K–10 K was chosen. A separate assembly process was done for each seed length. The HGAP (hierarchical genome assembly process) was done as follows; (1) Reads shorter than the seed length were aligned with the longer reads using BLASR. The errors on the long reads were corrected using aligned reads. (2) The high-quality corrected reads were assembled based on overlapping sequences to obtain a draft assembly. (3) All the reads were mapped to the draft assembly which polished the assembly to obtain the final genomic sequences. The seed length that gave the least contigs

were chosen as the final assembly. For plasmid sequences, we ran BLAST again itself to identify the redundant sequences at the end. The redundant sequences were clipped and the connected parts were re-checked by using PCR.

The *de novo* gene prediction on the plasmid sequences was performed by using CLC genomics work bench and GeneMarks (Besemer et al., 2001). The gene function was annotated by BLAST against Kyoto Encyclopedia of Genes and Genomes database KEGG pathway (Kanehisa et al., 2006). The chromosomal DNA sequence was deposited in the GenBank database under the accession number CP026636 and plasmid sequences were submitted under the accession numbers of CP026636-CP026640.

2.7. PCR confirmation of wild-type and plasmid cured halophilic strains

To differentiate wild-type *Bacillus filamentosus* HL2HP6 from plasmid cured HL2HP6-1, PCR amplification of chromosomal replication initiator protein DnaA gene, plasmid encoded genes, such as an integrase gene from plasmid pHL2HP6-1, a replication protein gene from plasmid pHL2HP6-2, a glutamate decarboxylase gene from plasmid pHL2HP6-3, a hypothetical protein gene from plasmid pHL2HP6-4 and plasmid pHL2HP6-5 were performed by using gene specific primers (Table 1). PCR reaction mixture contained 10 ng template DNA, 2.5 µL 10X *Taq* polymerase buffer (Fermentas), 1 µL 10 mM dNTPs (Fermentas), 2 µL of 25 mM $MgCl_2$ (Fermentas), 1 µL each of primers and 0.2 units *Taq* DNA polymerase (Fermentas) with total volume 25 µL. Amplification was performed in a Nyx Technik Amplitronyx (ATC201) Thermal Cycle with the following conditions; initial denaturation at 95 °C for 5 min, followed by 30 repeated cycles of 94 °C for 1 min, 52 °C for 50 s and 72 °C for 1 min and final extension at 72 °C for 5 min.

3. Results

3.1. Growth response of halophilic bacteria in salt supplemented medium

From the rhizosphere of *Salsola* and *Atriplex* and non-rhizospheric soils, more than 50% strains could tolerate up to 3.0 M NaCl while only few strains could grow up to the range of 3.5 and 4 M NaCl (Fig. 1A). More than 75% strains from all the soil samples were moderately halophilic and they required 1.5–2.5 M NaCl concentration for their optimum growth. Growth of most of the strains was better at 2 M NaCl and usually decreased with the increase in salt concentration (Fig. 1A).

3.2. Characteristics of plasmid cured halophilic strains

For each strain where plasmid curing was successfully obtained, one plasmid free segregate was selected and named after the name of their

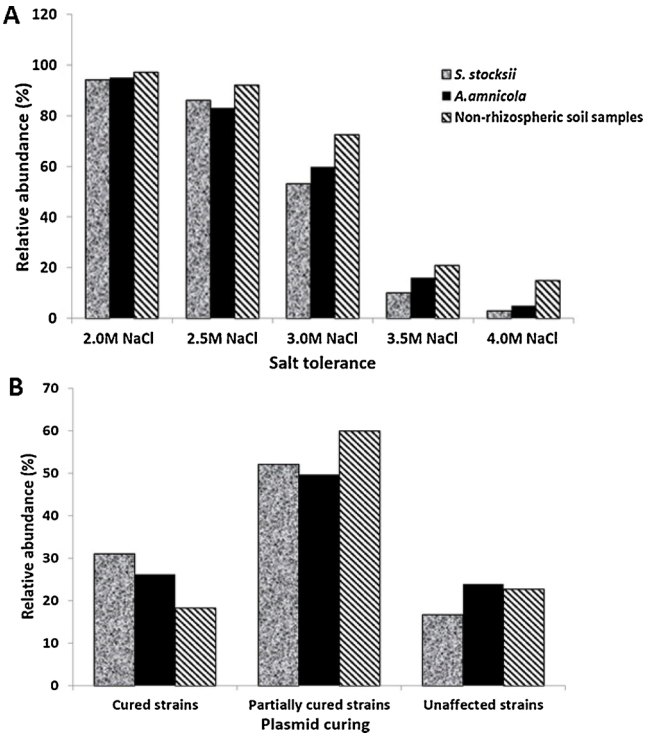

Fig. 1. (A) Growth response of halophilic bacteria isolated from the rhizosphere of *Salsola* and *Atriplex* and non-rhizospheric soils in salt supplemented medium, (B) Plasmid curing of halophilic bacterial strains from the rhizosphere of *Salsola* and *Atriplex* and non-rhizospheric soils.

strains, such as HL2HP6-1 (HL2HP6), AT2RP3-1 (AT2RP3), NRS2HaP1-1 (NRS2HaP1). Most of the bacterial strains were partially plasmid cured. These strains had genes for salt tolerance on their plasmid as well as on their chromosomal DNA. Bacterial strains from the *Salsola* rhizosphere showed maximum (31%) results for plasmid curing as compared to bacterial strains from other soil samples (Fig. 1B).

3.3. Isolation and restriction analysis of plasmids from halophilic strains

Plasmid size ranged approximately 7 to 105 kb. Most of the halophilic strains harbor more than 2 plasmids (Fig. 2A). These plasmids were designated on the name of bacterial and archaeal isolates as pHL2HP6 (HL2HP6), pAT2RP3 (AT2RP3), pNRS2HaP1 (NRS2HaP1). Plasmids from different halophilic strains (HL2HP6, AT2RP3, AT3HP4, NRS1HaP14, NRS2HaP1 and NRS3HaP12 were digested with *Eco*RI and *Bam*HI. Restriction patterns were similar in case of pHL2HP6, pAT2RP3 and NRS3HaP12 while the restriction pattern of other plasmids were

Table 1
Primer sequence and gene product used for confirmation of wild-type and plasmid cured halophilic strain HL2HP6.

DNA samples	Primer sequence (5'- 3')	Estimated gene amplification (bp)	Gene product
Genomic DNA	BeDnaAR: CGTTGTAATGTTGCCCAACAACAC BeDnaAF: CCGTGATTGGCTTGAGGCTCA	959	Chromosomal replication initiator protein DnaA
	BeHPF: GTGATACTCACGTCAGGAGAGGA BeHPR: GATGCACTGTCACCCATGTTTCC	630	Promoter for Chromosomal replication initiator protein DnaA
pHL2HP6-1	BeIntF: CCTTGCAAGGACTCCAGAATCACT BeIntR: CTGAGTCATTGAATGTGGAACTGC	618	Integrase
pHL2HP6-2	BeRypF: GAGTAGATTATCAGATTCGCACCGA BeRypR: AGTCGGGATGGTTCTGGTGCA	620	Replication protein
pHL2HP6-3	BeDccpF: TTACCACGGGTAAAGTCCATCAATG BeDeccpR: CTGGTCATGACAACCGGTAGGTTT	840	Glutamate decarboxylase
pHL2HP6-4	BeRepF: TGCTGTGGGTTTATTTGTCTATGCC BeRepR: GTATAAGGTACACAACCCTAGTTACC	1023	Hypothetical protein
pHL2HP6-5	BeDcpF: TGGGTGGGGAAGTTTCTAAGAACC BeDcpR: CGCAAATGAAGGCTATTTCGGTGA	1080	DUF3231 domain-containing protein

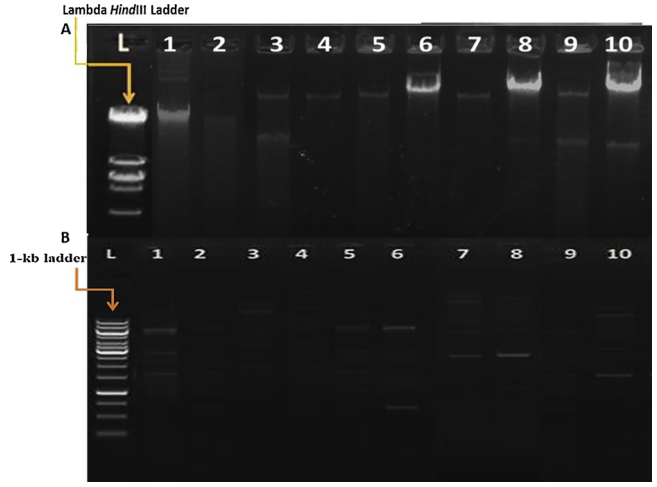

Fig. 2. (A) Plasmid isolation from the selected strains: In figure, Lane 1 = Lambda *Hind*III DNA Ladder; Lane 1–10 = plasmid DNA from different halophilic bacterial strains, (B) Selected plasmid DNAs digested with *Eco*RI and *Bam*HI: L = DNA marker, 1–10 = digested fragments of selected plasmids.

different from each other. The size of restiction fragments ranged from 750 bp to more than 10 kb (Fig. 2B).

3.4. Comparison of growth patterns of wild-type and cured halophilic strains and transformed E. colistrains

Plasmid DNA was transformed to *E. coli* strains DH10α and transformants were selected on HaP medium with 1.5 M NaCl concentrations. Growth behaviour of different wild-type and cured halophilic strains from the rhizosphere and roots of *Salsola* plants and transformed *E. coli* strains were compared by using HaP medium with 1.5 M NaCl (Fig. 3). Majority of the cured strains (HL1HP4, HL2HP6, AT1HP1 and NRS2HaP1) showed very little growth as compared to wild-type halophilic strains while partially cured strains (HL2RP7, AT2RP3 and NRS5HaP2) showed growth to some extant in HaP medium. Transformed *E. coli* strains with plasmids (pHL1HP4, pHL2HP6, pAT1HP1 and pNRS2HaP1) showed high populations as compared to transformed strains with plamids (pHL2RP7, pAT2RP3 and pNRS5HaP2). Wild-type *E. coli* strains showed no growth in HaP medium (1.5 M NaCl).

3.5. Antibiotics resistance profile of parental and plasmid cured halophilic strains

More than 90% of the wild-type halophilic strains showed resistance against at least 3 antibiotics (ampicillin, tobramycin and amoxicillin) while plasmid cured halophilic strains were sensitive to all these antibiotics (Table 2 and Fig. S2). Four wild-type halophilic strains HL1HP4, HL2RP7, HL2HP6 and NRS2HaP2 were resistant to ampicillin, tobramycin, amoxicillin and Sulfamethoxazol/Trimithoprim. Transformed *E. coli* strains with plasmids from the same four halophilic strains showed resistance against ampicillin and amoxicillin. Only 3 halophilic bacterial strains (AT1HP1, NRS2HaP1 and AT2RP3) showed resistance to penicillin and vancomycin (Table 2 and Fig. S2).

3.6. General features of genomic DNA of Bacillus filamentosusHL2HP6

The size of *Bacillus filamentosus* HL2HP6 was 5.18 Mbp with GC content of 36.81 and 5364 protein coding genes (Table S2 and Fig. 4). The genome of *Bacillus filamentosus* HL2HP6 were compared with *Bacillus endophyticus* Hbe603 and *Bacillus megaterium* QM B1551 genomes. A lot of small proteins detected were annotated as hypothetical proteins. The functional analysis of these proteins using KEGG pathway database showed that they have an important role in abiotic stresses and bioremediation of different toxic compounds. Different proteins and enzyme involved in various metabolic pathways were compared among 3 *Bacillus* strains HL2HP6, Hbe603 and QM B1551 using COG (clusters of orthologous groups) classification (Fig. 5). In the *Bacillus filamentosus* HL2HP6 genome, the number of genes related to cell division and chromosome partitioning (D), lipid metabolism (L), carbohydrate transport and metabolism (G), signal transduction mechanisms (T), defense mechanisms (V) and posttranslational modification, protein turnover (O) is higher than that is in the other two Bacillus strains while the number of genes related to amino acid transport and metabolism (E), cell membrane biogenesis (M), translation, ribosomal structure and biogenesis (I) and Intracellular trafficking and secretion (U) is lower as compared to that is in other two strains. Overall *Bacillus filamentosus* HL2HP6 genome has some distinctive features with regard to osmoregulation and abiotic stress tolerance. This strain is more similar to *Bacillus endophyticus* Hbe603 than the *Bacillus megaterium* QM B1551 (Fig. 5).

3.7. Functional analysis of plasmid sequences from Bacillus filamentosusHL2HP6

Results of whole genome sequence analysis showed that there were 5 plasmids in *Bacillus filamentosus* strain HL2HP6. The size of pHL2HP6-1 was 24.8 kbp, pHL2HP6-2 was 43 kbp, pHL2HP6-3 was 106 kbp, pHL2HP6-4 was 58 kbp and pHL2HP6-5 was 55 kbp (Fig. 6). Phylogenetic analysis showed that plasmids pHL2HP6-2 and pHL2HP6-4 had maximum homology about 79% (Fig. S3).

Sequence analysis of plasmid pHL2HP6-1 showed that a total of 23 gene coding sequences were identified. KEGG metabolic pathway analysis of these genes using showed that 17% genes involved in xenobiotics biodegradation and metabolism, 17% genes coded different proteins and enzymes which caused human diseases, 9% genes coded enzymes which involved in amino acid metabolism, 9% genes involved in energy metabolism, 8% genes in carbohydrate metabolism, 8% genes in metabolism of vitamins and cofactors and 8% genes in lipid metabolism (Table S3 and Fig. 6A).

Sequence analysis of plasmid pHL2HP6-2 showed that a total of 33 gene coding sequences were identified. Functional analysis of these genes using showed that they coded different proteins and enzymes which are known to be involved in genetic information processing (31%), amino acid metabolism (31%), enzymes families (7%), xenobiotics biodegradation and metabolism (8%), glycan biosynthesis and metabolism (8%) and human diseases (8%) (Table S4 and Fig. 6B).

A total 94 gene coding sequences were identified on the plasmid pHL2HP6-3. About 20% of these genes encoded proteins involved in human diseases (antibiotic resistance) and 20% with xenobiotics biodegradation and metabolism (Table S5 and Fig. 6C). Enzymes and other proteins that function in energy metabolism (10%), environmental information processing (10%), glycan biosynthesis and metabolism (10%), biosynthesis of other secondary metabolites (10%), metabolism carbohydrate metabolism (10%) were also encoded by this plasmid (Table S5 and Fig. 6C).

Plasmid pHL2HP6-4 encoded a total 46 genes that have functions in genetic information processing (35%), antibiotic resistance (17%), xenobiotics biodegradation and metabolism (12%), glycan biosynthesis and metabolism (6%), enzyme families (6%), cellular processes (6%) and environmental information processing (6%) (Table S6 and Fig. 6D).

Sequence analysis of plasmid pHL2HP6-5 showed that a total of 51 gene coding sequences were identified. Functional analysis showed that these genes coded different proteins and enzymes which are known to be involved in genetic information processing (25%), amino acid metabolism (20%), energy metabolism (10%), enzyme families (10%), xenobiotics biodegradation and metabolism (10%) and carbohydrate metabolism (5%) (Table S7 and Fig. 6E).

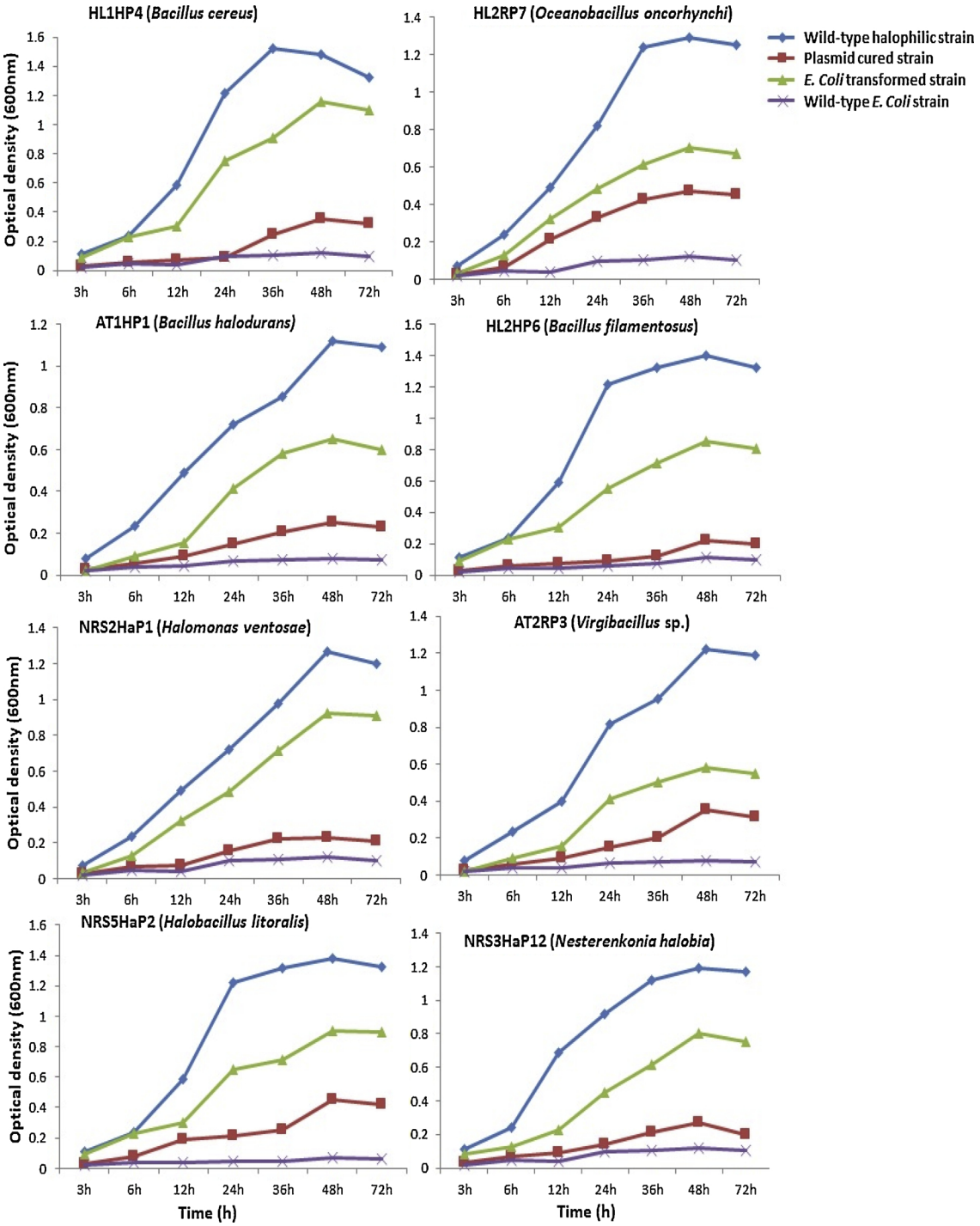

Fig. 3. Comparison of growth patterns of wild-type and plasmid cured halophilic strains, transformed and wild-type *E. coli* strains using HaP medium with 2 M NaCl concentration.

3.8. Prediction of proteins and enzymes involved in osmoregulation

A number of plasmids encoded osmoregulatory proteins and enzymes were identified using KEGG pathway analysis. Plasmid pHL2HP6-1 encoded proteins and enzymes including periplasmic proteins mdoA and mdoB, porins OmpA and OmpB, peptidases, 3-haloacid dehalogenase, peptidoglycan DL-endopeptidase, trehalases and integrase (Table 2). Plasmid pHL2HP6-2 encoded pyrroline-5-carboxylate reductase, glutamate synthase, proline dehydrogenase, fumarases, transcription factors abrB, malate dehydrogenase, putative transposase, Mn-containing catalase and organic acid transporters (Table 3). Plasmid pHL2HP6-3 encoded different proteins and enzymes including trehalose synthetase, cellobiose dehydrogenase, porins OmpC and OmpF, 2-haloacid dehalogenase, cysteine peptidases, K^+ transporters, phosphofructokinase and multidrug resistance, efflux pump AbcA (Table 3). Plasmid pHL2HP6-4 encoded proteins and enzymes including permeases, ProP and ProU, threonine peptidases, peptidoglycan biosynthesis and degradation amidase, EctB, EctC, ectoine synthetase, and MinD / ParA class of bacterial cytoskeletal proteins (Table 3). Plasmid pHL2HP6-5 encoded proteins and enzymes, including choline-sensing repressor protein, alanine dehydrogenase, choline dehydrogenase, choline kinase, putative lipoprotein and electrochemical potential-driven transporters (Table 3).

Table 2
Antibiotic resistance profile of wild-type and plasmid cured halophilic and transformed *E. coli* strains from the rhizosphere of halophytes (*Salsola* and *Atriplex*) and non-rhizospheric soil samples.

Bacterial strains		Antibiotics						
		AMP (30 µg)	TOB (10 µg)	AM (10 µg)	E (15 µg)	SXT (25 µg)	P (10 µg)	VA (30 µg)
HL1HP4	Wild-type halophilic strain	R	R	R	S	S	S	S
	Plasmid cured halophilic strain	S	S	S	S	S	S	S
	Transformed *E. coli*	R	S	R	S	S	S	S
HL2RP7	Wild-type halophilic strain	R	R	R	S	R	S	R
	Plasmid cured halophilic strain	S	S	S	S	S	S	S
	Transformed *E. coli*	R	S	R	S	S	S	S
HL2HP6	Wild-type halophilic strain	R	R	R	S	R	S	S
	Plasmid cured halophilic strain	S	S	S	S	S	S	S
	Transformed *E. coli*	R	S	R	S	S	S	S
AT1HP1	Wild-type halophilic strain	R	S	S	R	S	S	R
	Plasmid cured halophilic strain	S	S	S	R	S	S	S
	Transformed *E. coli*	R	S	R	S	S	S	R
NRS2HaP1	Wild-type halophilic strain	R	S	R	R	S	S	S
	Plasmid cured halophilic strain	S	S	S	S	S	S	S
	Transformed *E. coli*	R	S	S	S	S	R	S
AT2RP3	Wild-type halophilic strain	S	R	R	S	R	S	R
	Plasmid cured halophilic strain	S	S	S	S	S	S	S
	Transformed *E. coli*	S	R	R	S	S	S	S
NRS5HaP2	Wild-type halophilic strain	R	R	R	S	R	S	S
	Plasmid cured halophilic strain	S	S	S	S	S	S	S
	Transformed *E. coli*	R	S	R	S	S	S	S
NRS3HaP12	Wild-type halophilic strain	R	S	R	R	S	R	S
	Plasmid cured halophilic strain	S	S	R	R	S	S	S
	Transformed *E. coli*	R	S	S	S	S	R	S

Note: AMP = Ampicillin; TOB = Tobramycin; AM = Amoxicillin; E = Erythromycin; SXT = Sulfamethoxazol/Trimithoprim; P = Penicillin; VA = Vancomycin; R = Resistance; S = Sensitive.

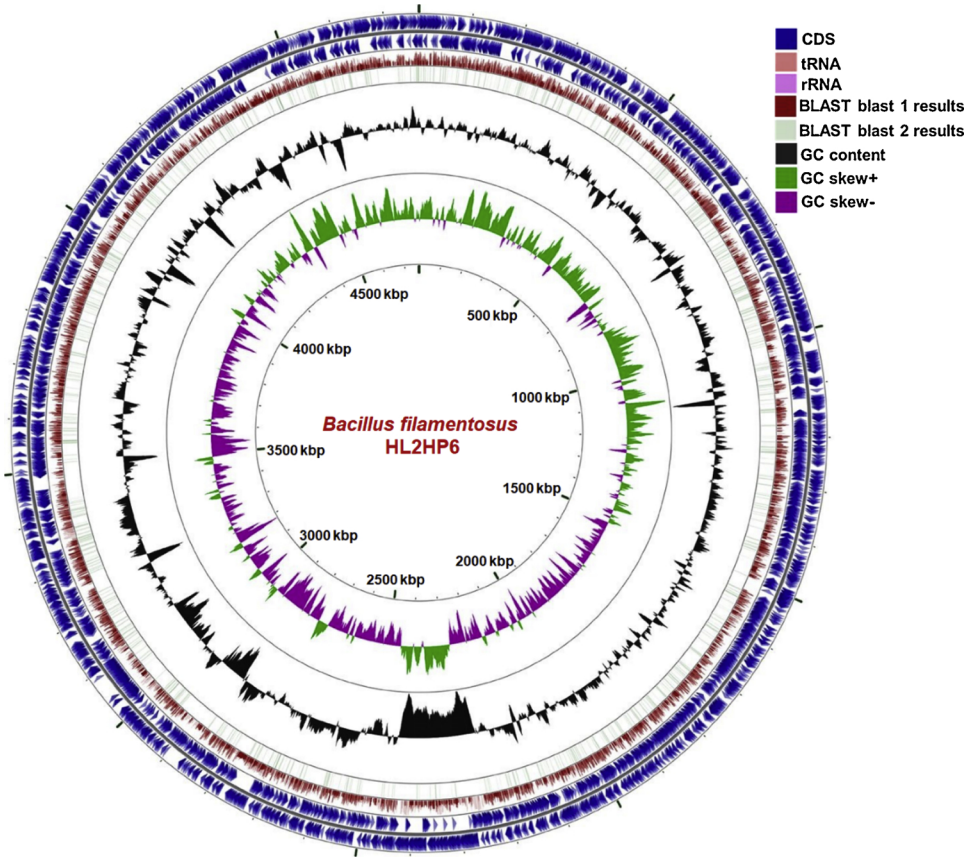

Fig. 4. Circular genome visualization of *Bacillus filamentosus* HL2HP6. Circles from the outside to the inside show the positions of protein-coding genes (blue), tRNA genes (red), rRNA genes (purple) on the positive and negative strands (Circle 1 and 2). Circle 3 and 4 showed the positions of BLAST hits with the genomes of *Bacillus endophyticus* Hbe603 and *Bacillus megaterium* QM B1551. Circles 5 and 6 show plots of GC content and GC skew plotted as the deviation from the average for the entire sequence.

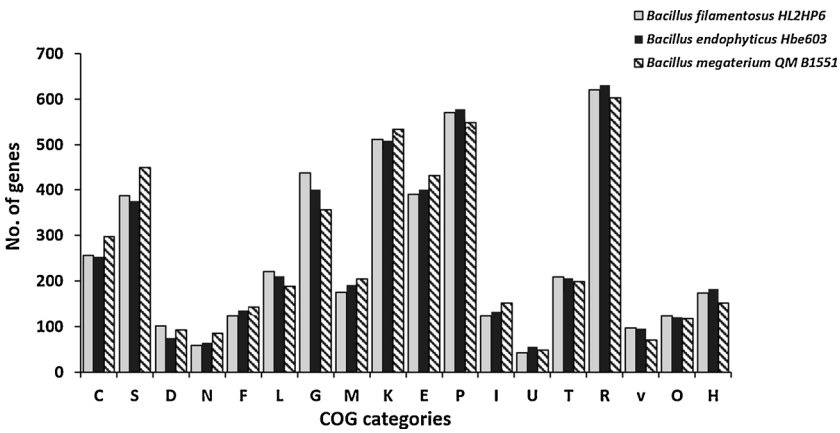

Fig. 5. COG analysis of *Bacillus filamentosus* HL2HP6 with other *Bacillus* species.
COG designation are presented as: C, Energy production and conversion; S, Function unknown; D, Cell division and chromosome partitioning; N, Cell motility and secretion; F, Nucleotide transport and metabolism; L, Lipid metabolism; G, Carbohydrate transport and metabolism; M, Cell membrane biogenesis; K, Transcription; E, Amino acid transport and metabolism; P, Inorganic ion transport and metabolism; I, Translation, ribosomal structure and biogenesis; U, Intracellular trafficking and secretion; T, Signal transduction mechanisms; R, General function prediction only; V, Defense mechanisms; O, Posttranslational modification, protein turnover and H, Secondary metabolite biosynthesis, transport and catabolism.

3.9. PCR confirmation of wild-type and plasmid cured HL2HP6 strain

To differentiate wild-type *Bacillus filamentosus* HL2HP6 from plasmid cured HL2HP6-1, PCR amplification of chromosomal replication initiator protein DnaA gene, plasmid encoded genes, such as an integrase gene from plasmid pHL2HP6-1, a replication protein gene from plasmid pHL2HP6-2, a glutamate decarboxylase gene from plasmid pHL2HP6-3, a hypothetical protein gene from plasmid pHL2HP6-4 and plasmid pHL2HP6-5 were performed by using gene specific primers (Fig. 7). Genes encoded by chromosomal DNA and plasmid pHL2HP6-3 were amplified from both wild-type and plasmid cured strains while genes encoded by plasmids pHL2HP6-1, pHL2HP6-2, pHL2HP6-4 and pHL2HP6-5 showed positive results in case of wild-type strain while in plasmid cured strains, these genes were absent (Fig. 7). These results showed that out of 5, 4 plasmids were eliminated during plasmid curing process.

4. Discussion

Halophiles reside in the rhizosphere and inside plant roots play an important in halophyte growth under salinity stress environments. The main aim of this study was to identify plasmid-conferring osmoregulatory genes in rhizospheric bacteria isolated from the rhizospheric and non-rhizospheric soils of *Salsola* and *Atriplex* by using plasmid curing of halophilic strains and then plasmids from halophilic bacterial strains were transformed into *E. coli* strains (Top10 and DH10α). The current study is the first report of its kind that it deals with the identification of osmoregulatory genes from rhizospheric halophilic *Bacillus* strains through whole genome sequence analysis. Plasmids from halophilic *Bacillus filamentosus* HL2HP6 were sequenced by using whole genome sequencing technique and analyzed using KEGG pathway database to identify different proteins and enzymes with important roles in osmoregulation of this bacterium.

Halophilic bacterial strains from non-rhizospheric soil samples were the most salt tolerant as compared to strains isolated from the rhizosphere of *Salsola* and *Atriplex*. They could tolerate salt concentration up to 4 M NaCl. More than 50% from the rhizosphere of halophytes could grow in the presence of 3.0 M NaCl concentration. Only a few strains identified in this study were able to survive at 4 M NaCl concentrations. Moderate halophiles are able to grow at 1.5–3.5 M NaCl in liquid as well as solid medium. The extreme halophiles are able to grow at high salt concentrations (3.4–5.1 M NaCl). Molecular phylogenetic studies indicated a great diversity of halophilic bacteria and archaea in brines, marine environments and solar salterns (Mahmood et al., 2009; Mukhtar et al., 2016; Oren, 2002; Ventosa et al., 1989).

About 50% bacteria from the rhizosphere of halophytes and 60% from non-rhizospheric soils were partially plasmid cured. These results indicated that mostly halophilic bacteria contain osmoregulatory genes on their chromosomal DNA as well as on their plasmids. Only a few bacterial strains completely eliminated their plasmids during curing experiments from all the soil samples. Plasmid conferring genes play an important role in flexibility and adaptation of abiotic stresses (Schuffler and Kübler, 2016). Elimination of native plasmids is usually an essential step in their analysis. Bacterial plasmids carry genes that codes for additional traits such as osmoregulation (Ohtani et al., 2016), CO_2 fixation, root nodulation and nitrogen fixation, antibiotic and heavy metal resistance (Ramirez et al., 2014) and plant tumor induction (Norberg et al., 2011a). However, the information about the prevalence and diversity of plasmids in bacteria from different environments is very limited.

Plasmid isolated from halophilic bacterial strains were approximately 7 to 125 kb in size. The detection of large plasmids is not usually easy because mostly plasmid isolation procedures based on disruptive techniques that permit a reliable extraction of small or moderate large plasmids ranging from 5 to 25 kb (Akinjogunla and Enabulele, 2010). Most of the *E. coli* transformants showed high transformation efficiency 69%, 75%, and 60% for plasmids isolated from the rhizosphere of halophytes (*Salsola* and *Atriplex*) and non-rhizospheric soils respectively. Plasmids from halophilic bacteria (*Halomonas, Marinobacter* and *Halobacillus*) encode genes involved in metabolism and osmoregulation that enabling their hosts to survive with unusual carbon and energy sources and under high salt conditions (Uddin et al., 2010). Qureshi and Malik (1990) presented evidence for plasmid mediated salt tolerance by suggesting the transfer of natural plasmids from a halophilic bacterium to non-halophilic *E. coli* K12 and a *Klebsiella* strain. Previous studies also showed the transfer of plasmid DNA from one bacterium to another during the transformation and conjugation experiments (Afrasayab et al., 2010; Galardini et al., 2013b).

More than 80% halophilic strains from the rhizosphere of *Salsola* and *Atriplex* and non-rhizospheric soils were resistant to ampicillin, tobramycin, amoxicillin and erythromycin while these strains were sensitive to sulfamethoxazol/trimithoprim, penicillin and vancomycin. Plasmids carry different genes that confer heavy metal resistance, antibiotic resistance, salt tolerance and enzymes that expand the nutritional ability of the cell (Bennett, 2010; Fukao et al., 2013). For marine environments, all of the extremely halophilic bacteria were resistant to different antibiotics, such as penicillin, streptomycin, tetracycline, vancomycin, kanamycin, chloramphenicol and erythromycin (Irawati et al., 2016; Woodford et al., 2011). The horizontal transfer of plasmids plays an important role in the dissemination of antibiotic resistance genes (Hall et al., 2016; Liangxing et al., 2016).

The whole genome sequence analysis of *Bacillus* HL2HP6 showed that more than 5000 protein coding genes were identified and a large number of small proteins detected were annotated as hypothetical proteins. The genes related to cell division and chromosome partitioning, lipid metabolism, carbohydrate transport and metabolism, signal transduction mechanisms, defense mechanisms, posttranslational modification, protein turnover, amino acid transport and metabolism,

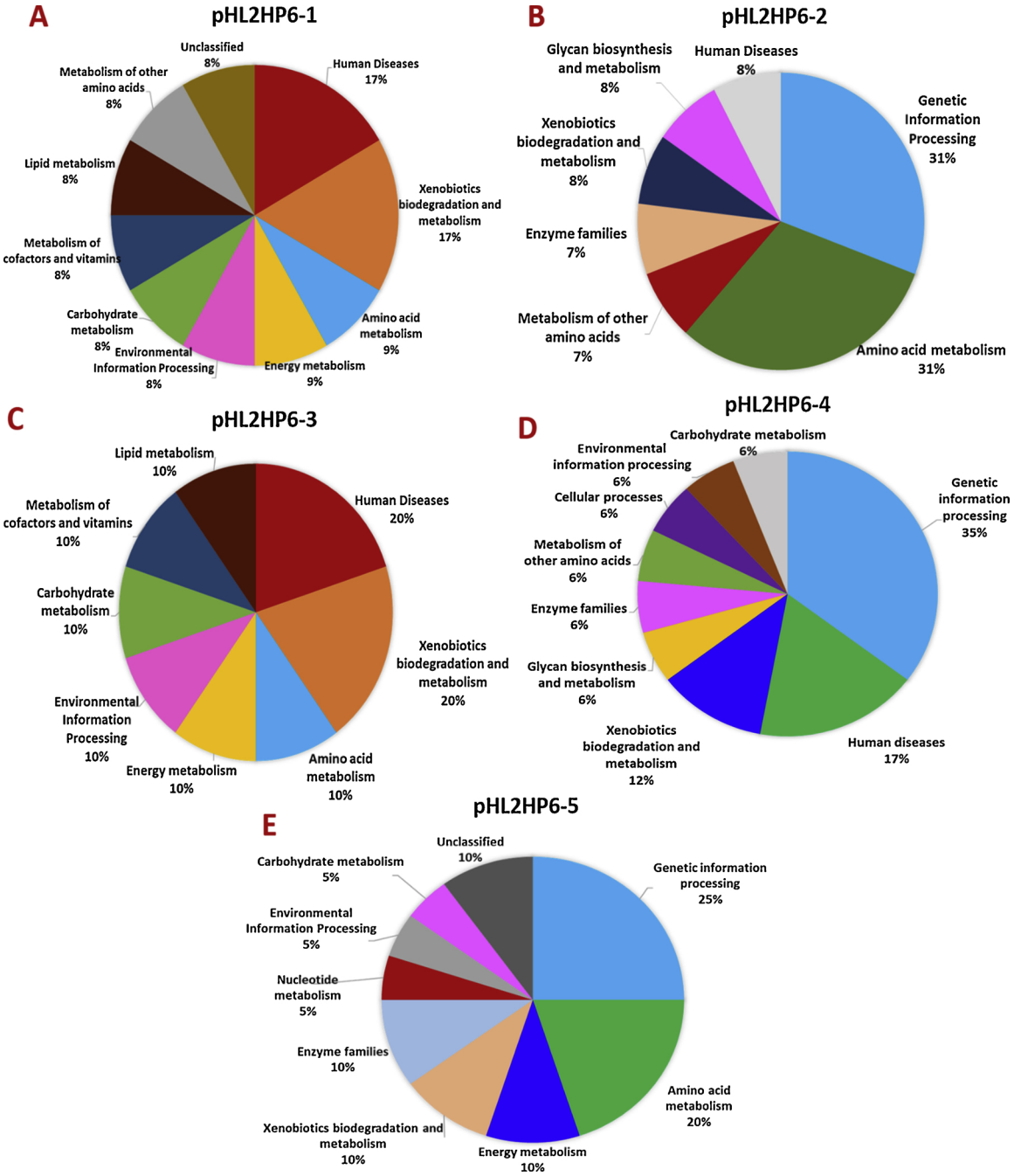

Fig. 6. Functional analysis of plasmids pHL2HP6-1 to pHL2HP6-5 encoded genes by using KEGG metabolic pathways.

cell membrane biogenesis, translation, ribosomal structure and biogenesis and intracellular trafficking and secretion were mainly identified through COG classification analysis (Zhao et al., 2013). Osmoregulatory genes for betaine, ectoine and proline were identified and characterized from halophilic *Bacillus, Halomonas, Virgibacillus, Halobacillus* and *Oceanobacillus* strains isolated from different environments (Mukhtar et al., 2018; Tanimura et al., 2016; Zou et al., 2016).

Sequencing of four larger and one megaplasmid from halophilic *Bacillus filamentosus* HL2HP6 showed that these plasmids encode different proteins and enzymes which are known to be involved in genetic information processing, carbohydrate metabolism, amino acid metabolism, enzyme families, xenobiotics biodegradation and metabolism, glycan biosynthesis and metabolism and human diseases. Some unclassified and uncharacterized proteins were also identified. Plasmids from halophilic bacterial strains carry complete sets of genes for transfer of plasmid DNA; the type 4 coupling protein (T4CP), xenobiotics biodegradation and metaboslism, origin of transfer (oriT), the relaxase protein and the type 4 secretion system (T4SS) (Bonfa et al., 2015; Brown et al., 2013; Shintani et al., 2015).

5. Conclusion

The current study is the first report of its kind that it deals with the identification of osmoregulatory genes from the rhizospheric halophilic *Bacillus* strain HL2HP6 through whole genome sequence analysis. More than 50% halophilic bacterial strains from the rhizospheric and non-rhizospheric soils were able to grow at 3 M salt concentrations. About 30% halophilic bacterial strains from the rhizosphere of halophytes and 20% halophilic bacterial strains from non-rhizospheric soils were completely plasmid cured. Most of the plasmid cured halophilic strains

Table 3
Detail of predicted enzymes and proteins involved in different metabolic pathways.

No. of enzymes /proteins	pHL2HP6-1	pHL2HP6-2	pHL2HP6-3	pHL2HP6-4	pHL2HP6-5
1	Periplasmic proteins mdoA and mdoB	Pyrroline-5-carboxylate reductase	Trehalose synthetase	Permeases, ProP and ProU	Choline-sensing repressor protein
2	Glutamyl kinase	Glutamate synthase	Cellobiose dehdrogenase	DABA aminotransferase	Alanine dehydrogenase
3	Porins, OmpA and OmpB	Putative lipoprotein	Porins, OmpC and OmpF	Threonine Peptidases	Choline dehydrogenase
4	Peptidases	N-acetylmuramoyl-L-alanine amidase	2-haloacid dehalogenase	Xenobiotic-transporting ATPase	Choline kinase
5	3-haloacid dehalogenase	Proline dehydrogenase	Cysteine peptidases	Peptidoglycan biosynthesis and degradation amidase	DABA aminotransferase
6	2,4-diaminobutyrate aminotransferase	Fumarases	K^+ transporters	DAB aminotransferase; EctB	Putative lipoprotein
7	Peptidoglycan DL-endopeptidase	Transcription factors abrB	RNA polymerase	Ectoine synthetase; EctC	Electrochemical potential-driven transporters
8	Trehalases	Malate dehydrogenase	Phosphofructokinase	N-acetylglutaminyl glutamine amide	Helix-turn-helix LacI family protein
9	Beta-Lactam resistance	Putative transposase	RNA-directed DNA polymerase	ABCB (MDR/TAP) subfamily	Mn-containing catalase
10		Mn-containing catalase		MinD / ParA class of bacterial cytoskeletal proteins	Beta-Lactam resistance
11	Integrase	RNA-directed DNA polymerase	Chromosome partitioning proteins	Beta-Lactam resistance	
12		Organic acid transporters	Multidrug resistance, efflux pump AbcA		

Fig. 7. PCR confirmation of **(A)** wild-type and **(B)** plasmid cured halophilic strains. Lane L = 1Kb DNA marker; Lane 1 and 2 = chromosomal replication initiator protein gene; Lane 3 = Integrase gene from plasmid HL2HP6-1; Lane 4 = Replication protein gene from plasmid HL2HP6-2; Lane 5= Glutamate decarboxylase gene from plasmid HL2HP6-3; Lane 6 = Hypothetical protein gene plasmid HL2HP6-4 and Lane 7 = DUF3231 domain-containing protein gene from plasmid HL2HP6-5.

indicated a change in antibiotic and metal resistance profile. Ability of transformed *E. coli* to grow on halophilic medium confirmed the presence of plasmid encoded osmoregulatory genes. Whole genome sequence analysis of *Bacillus* HL2HP6 showed the presence of a number of enzymes and other proteins involved in osmoregulation of microorganisms, such as trehalose, cellobiose inorganic ion transporters, ectoine synthetase, porins, proline, alanine, dehydrogenases and peptidases. The osmoregulatory genes identified in this study can be used to develop salt tolerant transgenic crops that can be grown in salinity affected areas.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126307.

References

Afrasayab, S., Faisal, M., Hasnain, S., 2010. Comparative study of wild and transformed salt tolerant bacterial strains on *Triticum aestivum* growth under salt stress. Braz. J. Microbiol. 41, 946–955.

Akinjogunla, O.J., Enabulele, I.O., 2010. Virulence factors, plasmid profiling and curing analysis of multidrug resistant *Staphylococcus aureus* and coagulase negative *Staphylococcus* spp. Isolated from patients with acute Otitis media. Am. J. Sci. 6, 1022–1033.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45 (4), 493–496 In this issue.

Bennett, P.M., 2010. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br. J. Pharmacol. 153, 347–357.

Besemer, J., Lomsadze, A., Borodovsky, M., 2001. GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. Nucl. Acids Res. 29, 2607–2618.

Bonfa, M.R.L., Grossman, M.J., Piubeli, F., Mellado, E., Durrant, L.R., 2015. Phenol degradation by halophilic bacteria isolated from hypersaline environments. Biodegradation 24, 699–709.

Brown, A., Benhar, I., Mizrahi, I., 2013. A method for purifying high quality and high yield plasmid DNA for metagenomic and deep sequencing approaches. J. Microbiol. Meth. 95, 272–279.

Culla, R.A., Diaz, M.R., Taylor, B.F., Roberts, M.F., 1999. Organic osmolytes in aerobic bacteria from Mono Lake, an alkaline, moderately hypersaline environment. Appl. Environ. Microbiol. 63, 220–226.

Dagla, H.R., Shekhawat, N.S., 2005. In vitro multiplication of *Haloxylon recurvum* (Moq.)-aplant for saline soil reclamation. J. Plant Biol. 7, 155–160.

DasSarma, S., DasSarma, P., 2015. Halophiles and their enzymes: negativity put to good use. Curr. Opin. Microbiol. 25C, 120–126.

Delmont, T.O., Eren, A.M., Maccario, L., Prestat, E., Esen, Ö.C., Pelletier, E., Le Paslier, D., Simonet, P., Vogel, T.M., 2015. Reconstructing rare soil microbial genomes using in situ enrichments and metagenomics. Front. Microbiol. 6, 358.

Durve, D., Naphade, S., Bhot, M., Varghese, J., Chandra, N., 2013. Plasmid curing and protein profiling of heavy metal tolerating bacterial isolates. Arch. Appl. Sci. Res. 5, 46–54.

Dziewit, L., Pyzik, A., Szuplewska, M., Matlakowska, R., Mielnicki, S., Wibberg, D., Artosik, D., 2015. Diversity and role of plasmids in adaptation of bacteria inhabiting the Lubin copper mine in Poland, an environment rich in heavy metals. Front. Microbiol. 6, 152.

El-Sayed, M., Helal, M., 2016. Multiple heavy metal and antibiotic resistance of *Acinetobacter baumannii* Strain HAF-13 isolated from industrial effluents. Am. J. Microbiol. Res. 4, 26–36.

Foo, Y.H., Gao, Y., Zhang, H., Kenney, L.J., 2015. Cytoplasmic sensing by the inner membrane histidine kinase EnvZ. Prog. Biophys. Mol. Biol. 118, 119–129.

Frage, B., Döhlemann, J., Robledo, M., Lucena, D., Sobetzko, P., Graumann, P.L., Becker, A., 2016. Spatiotemporal choreography of chromosome and megaplasmids in the *Sinorhizobium meliloti* cell cycle. Mol. Microbiol. 100, 808–823.

Fukao, M., Oshima, K., Morita, H., Toh, H., Suda, W., et al., 2013. Genomic Analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. PLoS One 8, e60521.

Galardini, M., Pini, F., Bazzicalupo, M., Biondi, E.G., Mengoni, A., 2013b. Replicon-dependent bacterial genome evolution: the case of Sinorhizobium meliloti. J. Evol. Biol. 5, 542–558.

Garcillan-Barcia, M.P., Alvarado, A., dela Cruz, F., 2011. Identification of bacterial plasmids based on mobility and plasmid population biology. FEMS Microbiol. Rev. 35, 936–956.

Hall, J.P.J., Wood, A.J., Harrison, E., Brockhursta, M.A., 2016. Source-sink plasmid transfer dynamics maintain gene mobility in soil bacterial communities. Proc. Natl. Acad. Sci. U. S. A. 113, 8260–8265.

Irawati, W., Yuwono, T., Rusli, A., 2016. Detection of plasmids and curing analysis in copper resistant bacteria *Acinetobacter*sp. IrC1, *Acinetobacter* sp. IrC2, and *Cupriavidus* sp. IrC4. Biodiversitas 17, 296–300.

Irshad, A., Ahmad, I., Kim, S.B., 2014. Cultureable diversity of halophilic bacteria in foreshore soils. Braz. J. Microbiol. 45, 563–571.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., et al., 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 34, 354–357.

Karan, R., Capes, M.D., DasSarma, S., 2012. Function and biotechnology of extremophilic enzymes in low water activity. Aquat. Biosyst. 8, 4–10.

Klumper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sorensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945.

Liangxing, F., Xingping, L., Liang, L., Shumin, L., Xiaoping, L., Jian, S., Yahong, L., 2016. Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from E. Coli isolates of food-producing animals. Sci. Rep. 6, 25312.

Mahmood, A., Latif, T., Khan, M.A., 2009. Effect of salinity on growth, yield and yield components in basmati rice germplasm. Pak. J. Bot. 41, 3035–3045.

Moghaddam, J.A., Boehringer, N., Burdziak, A., Kunte, H.J., Galinski, E.A., Schäberle, T.F., 2016. Different strategies of osmoadaptation in the closely related marine myxobacteria *Enhygromyxa salina* SWB007 and *Plesiocystis pacifica* SIR-1. Microbiology 162, 651–661.

Mukhtar, S., Mirza, M.S., Mehnaz, S., Malik, K.A., 2019. Isolation and characterization of halophilic bacteria from the rhizosphere of halophytes and non-rhizospheric soil samples. Braz. J. Microbiol. 50, 85–97.

Mukhtar, S., Mirza, M.S., Mehnaz, S., Mirza, B.S., Malik, K.A., 2018. Diversity of *Bacillus*-like bacterial community in the rhizospheric and non-rhizospheric soil of halophytes (*Salsola stocksii* and *Atriplex amnicola*) and characterization of osmoregulatory genes in halophilic Bacilli. Can. J. Microbiol. 64, 567–579.

Mukhtar, S., Ishaq, A., Hassan, S., Mehnaz, S., Mirza, M.S., Malik, K.A., 2017. Comparison of microbial communities associated with halophyte (*Salsola stocksii*) and non-halophyte (*Triticum aestivum*) using culture-independent approaches. Pol. J. Microbiol. 66, 375–386.

Mukhtar, S., Mirza, M.S., Awan, H.A., Maqbool, A., Mehnaz, S., Malik, K.A., 2016. Microbial diversity and metagenomic analysis of the rhizosphere of Para Grass (*Urochloa mutica*) growing under saline conditions. Pak. J. Bot. 48, 779–791.

Nath, A., 2016. Insights into the sequence parameters for halophilic adaptation. Amino Acids 48, 751–762.

Norberg, E., Orrenius, S., Zhivotovsky, B., 2011a. Mitochondrial regulation of cell death: processing of apoptosis-inducing factor (AIF). Biochem. Biophys. Res. Commun. 396, 95–100.

Norberg, P., Bergström, M., Jethava, V., Dubhashi, D., Hermansson, M., 2011b. The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. Nat. Commun. 2, 272.

Ohtani, N., Tomita, M., Itaya, M., 2016. Curing the megaplasmid pTT27 from *Thermus thermophilus* HB27 and maintaining exogenous plasmids in the plasmid-free strain. Appl. Environ. Microbiol. 82, 1537–1548.

Oren, A., 2002. Diversity of halophilic microorganisms: environments, phylogeny, physiology and applications. Ind. J. Microbiol. Biotechnol. 28, 56–63.

Oren, A., 2012. Taxonomy of the family Halobacteriaceae: a paradigm for changing concepts in prokaryote systematic. Int. J. Syst. Evol. Microbiol. 62, 263–271.

Petrovic, U., Cimerman, N., Plemenitas, A., 2004. Cellular responses to environmental salinity in the halophilic black yeast Hortaea werneckii. Mol. Microbiol. 45, 665–672.

Purdy, K.J., Cresswell-Maynard, T.D., Nedwell, D.B., McGenity, T.J., Grant, W.D., Timmis, K.N., Embley, T.M., 2004. Isolation of haloarchaea that grow at low salinities. Environ. Microbiol. 6, 591–595.

Qureshi, J.A., Malik, K.A., 1990. Evidence for a plasmid conferring salt tolerance in the plant-root associated, diazotroph *Klebsiella* sp. NIAB-I. Biotechnol. Lett. 12, 783–788.

Rahube, T.O., Viana, L.S., Koraimann, G., Yost, C.K., 2014. Characterization and comparative analysis of antibiotic resistance plasmids isolated from a wastewater treatment plant. Front. Microbiol. 5, 558.

Ramirez, M.S., Traglia, G.M., Lin, D.L., Tran, T., Tolmasky, M.E., 2014. Plasmid-mediated antibiotic resistance and virulence in Gram-negatives: the *Klebsiella pneumonia* paradigm. Microbiol. Spectrum. 2, 1–15.

Schuffler, A., Kübler, C., 2016. Targeting plasmids: new ways to plasmid curing. In: Unden, G., Thines, E., Schüffler, A. (Eds.), Host - Pathogen Interaction: Microbial Metabolism, Pathogenicity and Antiinfectives. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim, Germany.

Shintani, M., Sanchez, Z.K., Kimbara, K., 2015. Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Front. Microbiol. 6, 242.

Tanimura, K., Matsumoto, T., Nakayama, H., Tanaka, T., Kondo, A., 2016. Improvement of ectoine productivity by using sugar transporter-overexpressing Halomonas elongate. Enzyme Microb. Technol. 89, 63–68.

Uddin, M.S., Jiti, Z., Saleh, M.A., Ekram, A., Sikdar, B., Zaman, S., 2010. Role of large plasmid DNA in salt tolerance of a novel strain *Gracilibacillus* sp. *GTY*. J. Bio-Sci. 18, 34–39.

Ventosa, A., Gutierrez, M.C., Garcia, M.T., Ruiz-Berraquero, F., 1989. Classification of *Chromobacterium marismortui* in a new genus, *Chromohalobacter* gen. nov, as *Chromohalobacter marismortui* comb. nov, nom. Rev. Int. J. Syst. Bacteriol. 39, 382–386.

Woodford, N., Turton, J.F., Livermore, D.M., 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol. Rev. 35, 736–755.

Youssef, N.H., Savage-Ashlock, K.N., McCully, A.L., Luedtke, B., Shaw, E.I., Hoff, W.D., Elshahed, M.S., 2014. Trehalose/2-sulfotrehalose biosynthesis and glycine-betaine uptake are widely spread mechanisms for osmoadaptation in the Halobacteriales. ISME J. 8, 636–649.

Zhao, S., Kumar, R., Sakai, A., Vetting, M.W., et al., 2013. Discovery of new enzymes and metabolic pathways by using structure and genome context. Nature 502, 698–702.

Zou, H., Chen, N., Shi, M., Xian, M., Song, Y., Liu, J., 2016. The metabolism and biotechnological application of betaine in microorganism. Appl. Microbiol. Biotechnol. 100, 3865–3876.