Comparative genome analysis of *Bacillus okhensis* Kh10-101^T reveals insights into adaptive mechanisms for halo-alkali tolerance

Pilla Sankara Krishna
University of Hyderabad
Sarada Raghunathan
University of Hyderabad
Shyam Sunder Prakash Jogadhenu (syamsunderp@yahoo.com)
University of Hyderabad School of Life Sciences

Research article

Keywords: Bacillus okhensis, alkaliphilic, halophilic, genome analysis, hydroxyl ion stress, sodium toxicity.

Posted Date: May 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-25204/v1

Abstract

Background: *Bacillus okhensis*, isolated from saltpan near port of Okha, India, was initially reported to be a halo-alkali tolerant bacterium.We previously sequenced it's 4.86 Mb genome, here we analyze its genome and physiological responses to high salt and high pH stress.

Results: *B. okhensis* is a halo-alkaliphile with optimal growth at pH 10 and 5% NaCl. 16S rDNA phylogenetic analysis resulted in habitat based segregation of 106 *Bacillus* species into 3 major clades with all alkaliphiles in one clade clearly suggesting a common ancestor for alklaliphilic *Bacilli*. We observed that *B. okhensis* has been adapted to survive at halo-alkaline conditions, by acidification of surrounding medium using fermentation of glucose to organic acids. Comparative genome analysis revealed that the surface proteins which are exposed to external high pH environment of *B. okhensis* were evolved with relatively higher content of acidic amino acids than their orthologues of *B. subtilis*. It posess relatively more genes involved in the metabolism of osmolytes and sodium dependent transporters in comparison to *B. subtilis.* Growth of *B. okhensis* is Na^+ dependent, with a minimum requirement of 4% NaCl at neutral pH but 0.5% NaCl is enough at pH 10. It tolerated sudden increase of salt concentration of its medium, and exhibited an elongated cellular phenotype. But, could not tolerate a sudden shift of pH from 7 to 11, and cell envelope got damaged, confirming the pH regulation through cell wall reinforcement is key to survival at high-pH condition. We observed that hydroxyl ions damage the cell, but not Na+ ions, becuase at high pH Na+ ions were not accumulated inside.

Conclusion: *B. okhensis* uses acidification of the external medium and pH dependent cell wall reinforcement to survive sodic environments. Interestingly, its growth is highly Na^+ dependent and the genome encodes for a high proportion of acidic amino acids in majority of surface proteins in comparison to their orthologues of *B. subtilis,* a direct evidence of adaptive evolution.

Background

Bacteria that can tolerate high pH and/or high salt are gaining importance due to their biotechnological applications in industry. Evolution of cellular and molecular mechanisms as an adaptation to high pH were well documented in alkaliphilic bacteria belonging to *Bacillus* genus. [1–3]. It is important to note that majority of these alkaliphilic *Bacilli* were observed to be halotolerant too [2]. High pH tolerant *Bacillus* species were found naturally in soil, salines, sodic environments and are also enriched in man-made alkaline environments like indigo dye plants and industrial effluents [2, 4, 5]. Extremely alkaliphilic *Bacillus* strains grow optimally around pH 9.5 and often exhibit growth at pH 11 and above [1, 6]. Such extreme-alkaliphilic species are being used in industrial production of alkaline resistant enzymes such as proteases, amylases, xylanases, cellulases, and pullulanases [4, 7]. Studies on physiological and molecular responses of such extreme halo-alkali tolerant species is important to get insights into high pH and high salt stress adaptations and would clarify the molecular evolution of extremozymes [2, 8, 9].

Complete genome sequences of alkaliphilic, *B. halodurans* C-125, *B. pseudofirmus* OF4 and *Oceanobacillus iheyensis* HTE831 were reported and analyzed to obtain molecular information about adaptations to survive in high pH and high salt conditions [10–12]. The cell wall plays a key role in protection of the cell from deteriorative effects caused by high salt and high pH conditions. In *Bacillus species* the cell wall is heavily reinforced with teichuronic acid, a negatively charged layer that encloses the peptidoglycan cell wall. In alkaliphilic *Bacillus* species, these acidic polymers were reported to occur at a higher density when grown in an alkaline pH than in neutral pH [13, 14]. This anionic environment created by the negative charges of the polymers is hypothesized to repel hydroxyl ions and adsorb sodium and hydrogen ions, resulting in a passive external mechanism that contributes to survivability in alkaline environments [14].

The mechanism by which oxidative phosphorylation is achieved in alkaliphiles differs from neutrophilic bacteria due to the low proton-motive force across the cytoplasmic membrane. In alkaliphilic bacteria, the proton-translocating subunits *a* and *c* of the ATP synthase have specific sequence motifs to guard against cytoplasmic proton loss during ATP synthesis at high pH [15–18]. In extreme alkaliphiles the rotor forming c-subunit of the F_0F_1-ATP synthase contains AXAXAXA and PXXEXXP motifs in the N- and C-terminal helices, respectively [17]. Whereas, in neutrophilic *B. subtilis*, rotor forming c-subunit of the F_0F_1-ATP synthase contains GAGIGNG and ALVEALP motifs. Substitution of amino acid G by A in the N-terminal motif resulting in AXAXAXA was reported to play role in high pH tolerance [17]. A region in the external hydrophilic cytochrome c-binding domain is significantly more acidic in alkali-tolerant *Bacilli* than in neutrophilic *Bacillis*. This replacement of basic residues with neutral or acidic residues is commonly found in regions of the proteins that are on the exterior of the cell wall, exposed to the highly alkaline environment. The haloalkaliphiles were shown to produce high levels of catalase to eliminate oxidative stress and hydrogen peroxide produced during respiration [6, 19].

Tolerance to a high pH greatly depends on the cell's ability to maintain stable cytoplasmic pH [2, 20]. Alkaliphilic *Bacilli* from sodic soils or alkaline environments must survive under hyper osmotic stress and sodium toxicity as well. Osmotic stress greatly over laps with high pH stress in *Bacilli* due to significant increase in the Na^+ cytotoxicity due to high levels of Na^+ in their natural habitats [20], explaining the reason why in general alkaliphilic *Bacillus* species are halotolerant too. *Bacilli* that have adapted to high pH are generally believed to rely heavily on dedicated, highly effective Na^+/H^+ antiporters to grow and survive in alkaline habitats [21]. In alkaliphilic and alkali tolerant *Bacilli*, protons (H^+) from the external environment are transported into the cell in exchange with sodium ions (Na^+), thus acidifying the cytoplasm [22].

An extensive literature search suggests that the key changes that allow the bacteria to adapt halo-alkali stress are cell wall modifications, the ability to maintain stable cytoplasmic pH to near neutral pH, changes in the respiratory complexes to function in high external pH, synthesis of osmolytes, dedicated highly effective Na^+/H^+ antiporters, K^+ symporters for import of osmo-protectants and ability to maintain structural stability of proteins [1, 2, 13, 14].

Bacillus okhensis strain Kh10-101^T (here after *B. okhensis*) is a Gram-positive, strictly aerobic, rod like bacterium, first isolated from a salt pan in India, near the port of Okha by Nowlan et al., (2006) [23]. In this work, we analyzed the physiological response of *B. okhensis* to high pH and high NaCl conditions together with a comparative genome analysis with other halo-alkaliphilic species *B. pseudofirmus* OF4, *B. halodurans* C-125, *B. alcalophilus* ATCC27647, *B. clausii* KSM-K16 and a neutrophilic, *B. subtilis* subsp. *subtilis* strain168 from a halo-alkali tolerance standpoint. The comparative genome analysis was performed to differentiate the high salt responsive mechanisms from the high pH mechanisms and the hydroxyl ion (OH^-) effects from sodium (Na^+) toxicity. Our findings reveal the insights into halo-alkali tolerance mechanisms and emphasize for the first-time hydroxyl ion stress in high pH condition other than sodium toxicity.

Results

B. okhensis is a halo-alkaliphile

B. okhensis was originally identified as a halo-alkali tolerant bacterium [23], in our laboratory conditions optimal growth was observed when *B. okhensis* was allowed to grow in CMB medium with pH adjusted to pH 10 and 5% NaCl (Fig. 1A). Therefore, pH 10 and 5% NaCl concentration were used in all experimental conditions unless otherwise specified. Growth was observed with varying growth rates in NaCl concentrations ranging from 0.5 to 12% at optimal pH (pH 10). Similarly, growth was observed in media with a pH ranging from 7 to 11, when grown in the optimal NaCl concentration (5% NaCl). When the culture was grown in the medium containing a high salt (12 % NaCl) concentration and optimal pH, it exhibited an extended lag-phase (Fig. 1A). In contrast, when grown at low salt concentration (0.5% NaCl) at optimal pH the culture reached to stationary phase in ~6 h and a rapid decline in the cell density was observed afterwards, clearly demonstrating requirement of NaCl (5%) in the medium for stable cell density (Fig. 1A). Whereas, when cells were grown at a neutral pH (pH 7) and optimal salt concentration, final cell density was relatively low compared with that of the optimal growth conditions (Fig. 1A). A lag phase of 4-5 h was observed at high pH (pH 11) and optimal salt concentration with a slower growth rate ultimately reaching a cell density similar to that of optimal conditions during stationary phase (Fig. 1A). The data suggests that *B. okhensis* is a moderate halophile with minimum salt being essential for survival and optimal growth at 5% of NaCl. Interestingly it is also an alkaliphile with high final cell density at alkaline pH (pH 10) when compared with neutral pH growth conditions (Fig. 1A).

Cell morphology was examined using SEM imaging in the same growth conditions as mentioned above with cells were collected during the logarithmic growth phase. Cells grown at optimal conditions exhibited an average cell length of 3.5±0.6 µm with an average cell thickness of 0.87µm (Fig. 1B&C). When cells were grown in neutral or at high pH conditions, there was no significant difference in the cell morphology with an average cell length of 3.49±0.6 µm and 3.7±0.9 µm respectively (Fig. 1B, D&E). However, when grown in low salt concentration at an optimal pH, cells were smaller than that of the cells grown in optimal growth conditions, with an average cell length of 2.7±0.6 µm (Fig. 1B&F). In contrast, cells grown

in high salt at the optimal pH exhibited an elongated phenotype with an average cell length of 5.4±1.5 µm (Fig. 1B&G).

Phylogenetic analysis of *B. okhensis*

In order to identify the phylogenetic relationship of *B. okhensis* with other extremophilic *Bacilli*, we have generated a phylogenetic tree using 16S rRNA maximum likelihood phylogeny. 16S rDNA, bootstrap consensus phylogenetic tree of 106 *Bacillus* spp., has exhibited 3 major clades (Fig. 2). A small clade formed with 7 species consisted of well-known thermotolerant organisms. Among these *B. methanolicus* MGA3, *B. alveayuensis* 24KAM51, *B. smithii* DSM 4216, and *B. thermotolerans* SGZ-8were known to thrive at temperatures ranging from 50^{o}C to 55^{o}C. A second clade consisted of 27 species, which includes alkaliphilic and alkalitolerant *Bacillus* species. Interestingly except two, namely *B. coagulans* and *B. acidiproducens,* all other species in this clade were reported as either alkaliphilic or alkalitolerant species. This clade is subdivided into two subgroups, except *B. aidingensis* DSM 18341 and *B. kribbensis* DSM 17871, all other 20 species in this branch are known alkaliphiles falling into one subgroup (Fig. 2). The largest clade with 72 species contained all neutrophilic *Bacilli*. It is important to note that segregation of alkaliphiles and neutrophilic *Bacilli* confirms evolution of alkaliphiles from a possible common ancestor indicated with an arrow (Fig. 2).

General features of *B. okhensis* genome

Earlier, we reported the draft genome sequence of *B. okhensis* [24]. Its genome is of 4,865,284 bp with 38.2% G+C, 4,952 predicted CDS, 157 tRNAs, 8 rRNAs and 2 CRISPR arrays. To gain a deeper insight into the molecular mechanisms used by *B. okhensis* for its survival under extreme alkaline and saline conditions, the genomic features of *B. okhensis* were compared with the well-studied extreme alkaliphilic *Bacillus* model strains. In particular, the *B. okhensis* was compared with *B. pseudofirmus*, *B. halodurans, B. alcalophilus, B. clausii* and with *B. subtilis,* a neutrophilic non-halophile. The genome of *B. okhensis* is relatively large with significantly high number of predicted CDS than the other *Bacilli* used in this work (Table 1). However, *B. okhensis* has a similar G+C content to that of *B. pseudofirmus,* but not *B. halodurans*. The characteristic motifs of c-subunit of F_0F_1 ATP synthase which are known to guard against cytoplasmic proton loss at high pH were also present in *B. okhensis.* However, the N-terminal motif of *B. okhensis* contained GXSXAXA aminoacid sequence instead of ideal AXAXAXA motif (Table 1). The differences in the genome features of *B. okhensis* with that of other alkaliphiles and a neutrophilic *B. subtilis* and availability of genome sequencing data for several *Bacillus* species prompted us to perform comparative genome analysis to get insights into halo-alkali tolerant mechanisms among *Bacillus* species.

Bacillus okhensis contains genes for acidification of external environment and reduces the external medium pH:

Changes in the pH of external medium and growth of *B. okhensis* were monitored together after inoculation of the culture into the medium having different combinations of pH and NaCl concentration.

Interestingly, in CMB medium with glucose as carbon source, the pH of the external medium decreased with an increase in cell density, irrespective of the starting pH of the growth medium. When cells were inoculated at optimal conditions (pH 10 and 5% NaCl) in CMB medium, within 60 min, pH of the medium was reduced to ~9.5 with a simultaneous growth of the cultures and reached to pH 6 at stationary phase (Fig. 3A). However, when the culture was inoculated in a CMB medium at pH 11, no increase in the cell density was observed up to ~5 h after inoculation. During this long lag phase, pH of the medium got reduced gradually to ~pH 9.5 (Fig. 3B). Once the medium pH approached 9.5, the cell density started increasing. This indicates that when inoculated at high pH conditions, cells could be releasing organic acids to reduce the external medium pH before they actively start dividing. Interestingly, when inoculated at pH 7, the culture simultaneously started dividing with a concomitant decrease in the pH of the medium reaching to pH 6 in ~7 h post inoculation. However, the culture quickly reached stationary phase without producing much biomass; as the pH decreased further below pH 6.5 (Fig. 3C). The glucose consumption of cells grown in a high pH medium (pH 11) was monitored along with changes in the external pH of the medium. The amount of glucose decreased over time and reached a steady state after ~7 h inoculation (Fig. 3D). The decrease in the glucose levels in the external medium appeared to mimic the decrease in the pH of the medium, which reached pH 6.8 at stationary phase after 24 h (Fig. 3D). It is likely that the external medium pH was reduced by fermentation of glucose to acidic components. This was further confirmed by replacing glucose with non-fermentable malate as a carbon source. When cells were inoculated in a malate containing medium with an initial pH 9, no significant decrease in the pH of external medium was observed (Fig. 3E). Whereas with glucose, the pH of the medium was reduced to 6.2 from pH 9 after 24 h of incubation, confirming glucose fermentation is the source of the acidification of external medium (Fig. 3E).

The *B. okhensis* genome was compared with *B. subtilis* for genes involved in fermentation of carbohydrates to different organic acids, as it is clear from our data that the *B. okhensis* uses glucose to decrease the external pH of the medium. RAST subsystem features for fermentation and organic acid metabolism were presented in Table 2. The genome of *B. okhensis* encodes genes for metabolic pathways involved in the synthesis of organic acids, acetolactate, lactate, butyrate, acetoin, butane-diol and butanol. However, fermentation to acetolactate, butyrate and lactate were common in all the genomes compared in this study including *B. subtilis* (Table 2). Citrate metabolism and alpha aceto-lactate operon were observed to be present only in *B. subtilis* but not in alkaliphiles. The fermentation to mixed acids pathway is present only in *B. okhensis* and *B. alcalophilus* but not in the genome of *B. subtilis* and other alkaliphiles. The presence of fermentation to mixed acid pathway in *B. okhensis* makes it interesting as the pH of the growth medium is known to influence the type of fermentation product produced [25]. Thus, an active mixed acid pathway could be an important high pH responsive mechanism in *B. okhensis* and *B. alcalophilus* (Table S1). In *B. okhensis* with a predicted formate efflux transporter (WP_034625882.1), formic acid seems to be a potential exudate involved in pH reduction along with lactate, acetolactate and butyrate.

Adaptation of proteins for survival at extreme alkaline conditions in *B. okhensis*:

The pI and acid to base ratio of all the proteins from *B.okhensis* and *B.subtilis* were compared to analyse the changes that could have taken place during the course of adaptive evolution to cope up with the alkaline pH (Fig. 4). It is interesting to note that irrespective of their survival at alkaline or near neutral pH conditions, none of the proteins have pI near the biological pH, 7.5 in these two *Bacillus* species (Fig. 4A&B grey highlighted). Though *B. okhensis* thrives at alkaline habitat, it seemed to maintain the cytoplasmic pH close to near neutral pH, as none of the protein's pI value is in the range from pH 7 to 7.5. Majority of its cytosolic proteins have acid to base ratio and pI values comparable with *B. Subtilis* (Fig. 4A). Over all there is a slight shift in the pI curve of proteome of *B. okhensis* towards acidic region when compared to *B. subtilis* (Fig. 4B). Similar pattern of pI shift of proteins towards acidic region and lack of proteins with pI near probable cytoplasmic pH was observed in all alkaliphiles used in this study (Fig. S1). There are many proteins with pI in the acidic range in alkaliphile and alkaline range in *B. subtilis* (indicated with open and closed arrows respectively in Fig. 4B). Interestingly, about 122 proteins of *B.okhensis* showed significantly lower pI and acid to base ratios when compared to the corresponding orthologs from *B. subtilis* (Table S2, Fig. 4C). It is interesting that almost all the proteins with relatively low pI values in *B. okhensis*, but not in *B. subtilis* were surface proteins such as, cell wall, outer membrane, flagellar, spore formation related proteins and some hypothetical proteins. This demonstrates that, as these surface proteins are often being exposed to high pH and affected by the external environment than the cytosolic proteins, *B. okhensis* might have evolved with relatively higher acid to base ratio in the proteins during the course of adaptive evolution.

Role of cell envelope in halo-alkali tolerance

Genome of *B. okhensis* was searched for the presence of homologous genes of cell wall synthesis, which were reported to play an essential role in the pH homeostasis in *B. halodurans* C-125 [14, 26]. We found that the *B. okhensis* genome has 7 Teichuronic acid biosynthesis genes, whereas extreme alkaliphile *B. pseudofirmus,* which can tolerate sudden shifts from low to high pH has only 3 gene (Table 3). It has been reported that constitutively expressed genes for formation of S-layers contribute to the adaptability to sudden shifts to extreme alkalinity but are unfavorable to the growth of *B. pseudofirmus* OF4 at near-neutral pH [1]. Interestingly, RAST SEED has more orthologs and subsystem features annotated for genes responsible for capsular and extra cellular polysaccharide synthesis in neutrophilic *B. subtilis* than extreme alkaliphiles (Table 3). Surprisingly, genes coding for Murein biosynthesis integral membrane protein MurJ (WP_034630126.1) and MFS transport protein, YceL (WP_034626199.1) were identified in *B. okhensis* but not in any of the compared strains. In alkaliphiles the head group of the phospholipids in the membrane often consists of an array of branched chain negatively charged lipids, such as cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine. Of these, cardiolipin is the most important component contributing to pH homeostasis. A high concentration of cardiolipin in the plasma membrane is a characteristic feature of alkaliphilic *Bacilli* [27]. In the genome of *B. okhensis*, three genes coding for cardiolipin synthase (Cls), WP_034629090.1, WP_034631834.1 and WP_034631890.1 were identified. It is interesting to note that the *B. okhensis* genome harbors a pair of genes that encodes DesA (fatty acid desaturase WP_034627982.1) and acyl-CoA desaturase (WP_034628144.1). Even the genome of the extreme alkaliphile, *B. pseudofirmus* encodes a gene for fatty acid desaturase (WP_012957426.1).

However, it has been reported that the facultative alkaliphiles lack desaturase activity and incorporation of unsaturated fatty acids in the membrane restricts these organisms' ability to grow at near-neutral pH [28].

Adaptations for high salinity

Uptake of potassium (K^+) plays an important role, along with osmo-protectants such as glycine betaine, in salt stress tolerance [29, 30]. The *B. okhensis* genome contains genes coding for five different potassium channels, which include 4 potassium channels (WP_034633860.1, WP_034632084.1, WP_070808113.1, WP_034626537.1) and 1 potassium voltage gated channel (WP_034629238.1). It also has potassium efflux system, KefA, (WP_034627384.1), potassium uptake related transporter proteins KtrB and KtrD (WP_034627056.1; WP_034632305.184.1), potassium uptake protein KtrD (WP_034627056.1) and 3 copies of TrkA family potassium uptake proteins (WP_034632376.1, WP_034632856.1 and WP_034634053.1). Moderate halophiles may use organic solutes for osmotic balance rather than increasing internal ionic concentration as a response [30, 31]. The presence of a gene coding for a putative L-ectoine synthase (WP_034626156.1, Contig 3: 23712-24104 bp) was found in *B. okhensis* and also in other halotolerant *Bacilli,* but not in *B. subtilis*. *B. okhensis* has genes coding for choline and glycine-betaine metabolism. They are choline sulfatases (WP_034627928.1 and WP_034627930.1), DUF229 domain-containing proteins (WP_034627934.1, WP_052144881.1 and WP_052144652.1), Choline dehydrogenase (WP_034633942.1) and choline uptake protein, BetT (BCCT family transporter WP_034625673.1). In total, 15 genes are involved in glycine betaine metabolism, which includes 6 copies of glycine betaine *opuD* family transporters (WP_034624919.1, WP_034631193.1, WP_052144695.1, WP_034627402.1, WP_034627338.1 and WP_034626526.1), 7 copies of glycine betaine ABC transport system proteins (WP_034626805.1, WP_034633483.1, WP_034633484.1, WP_034633486.1, WP_034633941.1, WP_034626802.1 and WP_034626803.1). It has highest number of genes related to the glycine betaine transport when compared to the other species suggesting that it also relies on chemical osmo-protectants to thrive under extreme halo-alkaline conditions (Table 4).

The *B. okhensis* genome harbors several genes related to the production of sugar alcohols, such as mannitol-1-phosphate 5-dehydrogenase, (WP_034631367.1) 3 copies of Sorbitol dehydrogenase (WP_034625617.1, WP_034625836.1 and WP_084138909.1). Twenty genes were identified to be involved in inositol metabolism (EC. 1.1.1.18, EC 1.2.1.27 and EC 3.1.3.25) and myo-inositol-sodium symporter (WP_084138614.1). Thus, the *B. okhensis* genome has evolved with genes responsible for adaptation to high salt in its natural habitat, saltpan. The number of genes annotated and predicted to be involved in synthesis of different osmolytes and ectoine in different *Bacillus* strains is presented in Table 4.

Sodium dependent growth and survival of *B. okhensis* and pH homeostasis

Growth of *B. okhensis* in CMB medium with different sub-optimal NaCl concentrations was monitored. *B. okhensis* required a minimum of 4% NaCl to survive at neutral pH 7. When inoculated in a medium containing low salt (*i.e.,* 0.5 % NaCl) it is necessary to set a high pH medium for its growth, suggesting

sodium ion dependency for its survival (Fig.5). Without NaCl in the medium, growth was not observed at any pH (7 to 10) (Fig. 5A). However, when inoculated in a medium with a pH of 9 or 10 in the absence of NaCl, the maximum cell density obtained was very low and the same was decreased immediately showing absolute NaCl requirement for survival of *B. okhensis* (Fig 5A). With 0.5% NaCl in the medium, cells exhibited signs of growth at pH 10, but not in pH 7, 7.5, 8 or 9 (Fig. 5B). A slight increase in the cell density was observed at pH 9 but this was significantly lower than that of pH 10 (Fig 5B). Interestingly, even at pH 10 and 0.5% NaCl the cell density decreased after 7h of growth suggesting cell lysis due to insufficient Na^+/NaCl availability (Fig. 5B). In the presence of 2% NaCl in the medium, cells exhibited growth at pH 8, 9 and 10 but not at pH 7.5 or pH 7 (Fig. 5C). At a concentration of 2% NaCl, cell density was stable at pH 10, but not in pH 9 or pH 8 (Fig 5C). In the medium with 4% NaCl cells started growing in all the pH conditions tested, with a stable cell density being seen during stationary phase (Fig. 5D). These observations confirmed that a certain amount of Na^+ ions are essential for the growth and survival of *B. okhensis*. This sodium ion dependency of growth is expected to be due to the role of Na^+ in nutrient transport as well as high pH tolerance.

Since we observed that the sodium ions play a key role in growth of *B. okhensis,* we analysed the genome of *B. okhensis* for candidate genes involved in sodium dependent transport. Extreme alkaliphilic *Bacillus* strains are generally believed to rely heavily on dedicated, highly effective Na^+/H^+ antiporters by which, H^+ ions from the external environment are transported into the cell in exchange for sodium ions (Na^+), thus acidifying the cytoplasm, for growth and survival in alkaline habitats. The main mechanism of pH homeostasis in *B. okhensis* is probably catalysed by NhaC and NhaD antiporters (WP_034627995.1and WP_034625225.1). Interestingly, NhaD in *B. okhensis* has an ortholog in extreme alkaliphile *B. pseudofirmus* (84% identity) but not in other species compared. *B. okhensis* genome has 12 genes coding for Na^+/H^+ antiporter subunits which is similar in other *Bacillus* species (Table 4). In sodic environments cells require more energy to survive as they must expend more energy taking up solutes and extruding sodium ions [2, 20]. The requirement for robust Na^+ extrusion is most likely important because most of the ion-coupled solute symporters and flagellar motors of *B. okhensis* are Na^+ coupled. Indeed *B. okhensis* has 10 candidate genes predicted to be involved in sodium dependent transport of nutrients and 8 candidate genes involved in sodium-based symport of nutrients, whereas in *B. subtilis* only 4 were present (Table 4). Molecules that are transported in a sodium ion dependent manner include glutamine, glutamate, proline, phosphate, sulphate, bicarbonate and alanine. This family of transporter proteins function to exchange internal sodium ions with external solute molecules or these solutes are co-transported into cell using a sodium gradient. Moreover, the genome of *B. okhensis* has a sodium ion influx: the voltage-gated sodium channel (WP_052144823.1) and the sodium symporters and transporters (22 proteins). *B. pseudofirmus* OF4 mutants with no functional NaVBP fail to maintain pH homeostasis during sudden alkaline shifts in the external pH [32]. The NaVBP sodium channel of *B. okhensis* is closely related to that of *B. wakoensis* (81% sequence similarity) and exhibited no significant similarity with that of *B. pseudofirmus*, *B. halodurans* and *B. alcalophilus*. Na^+ re-entry in alkaliphiles can happen through the Na^+ coupled flagellar Mot complex, which functions as an ion channel while

converting chemiosmotic energy into mechanical energy. The *motPS* genes coding for Na^+ coupled Mot complex are mostly found in alkaliphiles, such as *B. pseudofirmus* OF4, *B. halodurans* C-125, and *O. iheyensis* HTE831 [32]. Whereas, proton coupled Mot complexes coded by *motAB* genes are widely distributed among *Bacillus* spp., including the alkaliphiles. The genome sequence of *B. okhensis* revealed only a single set of genes encoding a MotAB-like pair of proteins as the stator (flagellar motor protein MotP, WP_034629847.1 and flagellar motor protein MotA WP_034630220.1). These stator proteins are closely related to that of *B. wakoensis* and *B. akibai* with similarity of 86% and 85% respectively but not shown any significant similarity with that of *B. pseudofirmus* or with *B. halodurans* and has exhibited 36% identity with the MotA in *B. clausii*, which is known to change coupling ion from a proton to a sodium ion in response to external pH [32].

Response of *B. okhensis* to a sudden shift to high salt

Bacteria can cope up with a sudden increase in salinity by activating acclimation mechanisms. To investigate the effect of exposure to high salt sudden shift on the growth and cell morphology of *B. okhensis*, cells in exponentially growing phase at low salt (OD_{520}, 0.2) were shifted to high salt condition. Initially no increase in the cell density was observed for a period of 60 min after a sudden shift from 0.5 to 12% NaCl and then growth had resumed (Fig. 6A). As already mentioned, *B. okhensis* grows to low cell density without any stable stationary phase (Fig. 1A). But, shift of low salt (0.5%) acclimated cells to high (12%) salt led to higher cell density with stable stationary phase. The cell morphology of these high salt treated cells were examined using SEM. Cells were taken at 30, 60 and 180 min after the shift to 12% salt and compared to an untreated reference (Fig. 6B). Cells exhibited an elongated phenotype during high salt treatment (Fig. 6C, D and E). Within 30 min no significant difference in the cell size and shape was observed. After 60 min of high salt treatment, an increased number of cells exhibited elongated phenotype (Fig. 6D). After 1 hour of treatment, the cells began to divide resulting in a mix of elongated and short cells, confirming that some of the elongated cells started dividing after acclimation to high salt (Fig. 6E). In previously studied non-halotolerant *Bacillus* models, salt stress resulted in formation of filamentous cells due to inhibition of cell septum formation. This filamentous phenotype was attributed to inhibition of autolysins, involved in hydrolyzing peptidoglycans, which allows the daughter cells to separate during division [33]. Similarly, inhibition of cell septum formation might be involved in elongated phenotype in *B. okhensis* in response to high salt stress that was also observed in *B. cereus* [34]. In addition, putative cell division inhibitor (*yfcH* homolog) gene was identified (WP_034626153.1) in the genome of *B. okhensis*. Our observations suggest that sudden shift from low salt (0.5%) to high salt (12%) has not shown any lethality or significant damage of metabolism or cell growth and survival. It clearly indicates that the genome of *B. okhensis* has evolved with mechanisms to quickly adapt to sudden changes in external NaCl concentration.

High pH shock and Hydroxyl ion stress

Bacterial cells must maintain near-neutral cytoplasmic pH and devoid of sodium toxicity to survive in alkaline-sodic environment [2, 20, 22]. A sudden shift in pH from low to high will create alkaline shock or

hydroxyl ion stress on the cells. It was reported that *B. subtilis* showed no growth following a rapid pH increase [35]. *B. okhensis* cells those were pre-adapted to pH 7 were given a high pH shock by changing the pH of the medium to pH 11. This sudden shift in pH is followed by no increase in cell density for several hours (Fig. 7A), growth resumed after ~36 h of pH shock (results not shown). Even though, *B. okhensis* is a halo-alkaliphile and has evolved with adaptive mechanisms to tolerate high pH during the course of evolution, cells which were fully acclimated to pH 7 cannot tolerate the change to a high pH. It is likely that the organism might have turned off the adaptive mechanisms needed for growth under high pH conditions. After the shift of cultures from pH 7 to 11, the bacterial cells became wrinkled and severely damaged (Fig. 7B, C&D, Fig. S2). Such changes were not observed when pH 10 acclimated cells were shifted to high pH 11 (data not shown). This data clearly indicates that the all the mechanisms required for survival at pH 10 and above are operative during their growth, therefore a shift from pH 10 to 11 did not cause any cellular damage. When the culture from optimal growth conditions (pH 10 and 5% salt) was inoculated to pH 7 after several generations, it might have acclimatized to survive at pH 7, perhaps by turning off all high pH-tolerant mechanisms. Inoculation into pH 11 medium, from pre-cultures maintained in pH 9 or 10 mediums does not shown damage to the cell surface (Fig. 1E). This suggests that an alkaliphile could adapt to near neutral pH condition by switching off the mechanisms that are necessary for high pH tolerance. In *B. subtilis* transport of inorganic ions, polyamines and several other genes were upregulated upon high pH stress, which includes K^+/H^+ antiporters, sig-ω regulon[35]. Similar mechanisms might exist in *B. okhensis*. Indeed, upon a high pH shift both the *B. okhensis* membrane and cell wall were damaged.

Change in cellular Na^+/K^+, with salt stress and high pH stress

As observed in previous sections, a sudden change in the pH of the medium with NaOH caused severe cellular damage, but the same was not observed with high NaCl shock. This clearly indicates that at a high pH, cells have to deal with hydroxyl ions along with sodium toxicity. To differentiate the damage made to the cells by hydroxyl ions from that of sodium cytotoxicity, entry of sodium ions into the cells due to high pH challenge was evaluated. Cellular elemental composition was estimated using SEM assisted EDX (Fig. 8). SEM assisted EDX analysis has shown that the cellular content of *B. okhensis* grown in optimal conditions contained 32.9% Na^+ and 5.48% K^+ (Na^+/K^+ ratio 6) respectively. When grown at a neutral pH the Na^+/K^+ ratio was 0.91(13.8/15.1% relative units respectively). At 30 min after shifting to high pH the ratio increased to 16.09 (35.4/2.2). This clearly indicates a sudden shift in pH leads to increased cellular Na^+ ion concentration with a concomitant decrease in K^+ concentration. This rise could be because of damage to the membrane or an increase in Na^+ ions inside the cell due to change in equilibrium of the cellular sodium cycle. However, when cells were allowed to grow at 0.5% NaCl the Na+ / K+ ratio was 1.15 (14.4/12.5% relative units respectively). But at 30 min after a shift of cultures from low salt to high salt concentration (0.5 to 12%), the ratio was increased to 2.89 (34.2/11.8). From these observations, we conclude that cells accumulated similar amount of sodium ions, when shifted from 0.5 to 12% NaCl as well as upon a sudden shift from pH 7 to 11. This could be due to the damage caused by the excessive hydroxyl ions to the cell wall as observed under SEM (Fig. 6). It is not the entry of sodium

ions or sodium toxicity that caused the cellular damage during high pH stress, because EDX analysis has shown that shift to high pH led to no significant increase in the internal sodium ions in comparison with cells grown in optimal conditions. Therefore, the physical damage to the cell wall could be due to hydroxyl ion stress.

Genes coding for enzymes with potential applications in industry

Various alkaline enzymes, such as proteases, amylases, and cellulases, have been successfully commercialized on an industrial scale with a significant share in global enzyme industry [7, 36]. We analyzed genome of *B. okhensis* for enzymes of industrial importance. The genome of *B. okhensis* exhibited almost 40 different proteases which include members of the serine protease family of enzymes. Extracellular serine protease (Peptidase S8, WP_034631261.1) and alkaline serine protease (serine protease, WP_034628755.1) are potentially of industrial importance. Genome annotation of *B. okhensis* also shown 7 different genes encoding for lipases which includes industrially important enzymes, 4 copies of alpha/beta hydrolase, (WP_034628252.1, WP_034629946.1, WP_052144559.1 and WP_052144539.1) and lipase (lipase family protein, WP_084138891.1), hydrolase (WP_034627892.1) and lysophospholipase-like family protein (WP_034633601.1). Two Xylanases were identified (endo-1,4-beta-xylanase, WP_034628000.1 and 1,4-beta-xylanase, WP_034630757.1) and a Chitinase (WP_052144883.1). A total of 6 catalase related genes were found, homologs of catalase/peroxidases (WP_034625486.1 and WP_034630585.1) catalase (WP_034630685.1) and paralogs of Mn containing catalases (WP_034626999.1, WP_034624914.1 and WP_034631097.1) were found.

Discussion

The natural cause of sodic environments is the presence of soil minerals producing sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$). Therefore, saline stress and sodium toxicity overlaps with high pH stress in alkaliphilic *Bacillus*. *B. okhensis* reported as halo-alkalitolerant bacterium; is indeed a moderate halophile and an alkaliphile with growth optima falling at 5% NaCl and pH 10 in CMB medium. *B. okhensis* upon incubation in 12% NaCl exhibited elongated phenotype explaining probably high salt stress resulted in inhibition of daughter cells partition during cell division (Fig. 1G and Fig. 6D&E). Based on 16S rDNA sequence similarity *B. okhensis* is closely related to *B. wakoensis* and there is a possible common ancestor for all the alkaliphilic *Bacillus* spp. (Fig. 2). Interestingly *B. okhensis* has a relatively large genome with more CDS regions than its closest alkaliphilic models (Table 1). Genes for fermentation to organic acids was observed in all the species compared, however formate efflux is a possible high pH response observed in *B. okhensis* (Table 2&S1). Along with genes predicted for fermentation of glucose to organic acids acidification of external medium was demonstrated in *B. okhensis* which was further confirmed by using malate as carbon source (Table 2, Fig. 3A, B, C, D&E). Based on earlier reports of *Bacillus* cell wall composition and its relation to tolerance of high pH, no correlation was observed with pH tolerance and the number of genes predicted to be involved in cell wall synthesis or membrane composition. Extreme alkaliphilic bacterium *B. pseudofirmus*, or *B. okhensis* has only Teichoic acid (TA) biosynthesis and Teichouronic acid biosynthesis genes but alanyl-lipoteichoic

acid biosynthesis, sortase metabolism and polyglycerolphosphate and lipoteichoic acid metabolism genes are not detected (Table 3). Contrasting from previous reports from Dunkley et al., (1991) [28], a membrane desaturase gene was identified in *B. okhensis* and even in extreme alkaliphile *B. pseudofirmus*. Further biochemical and mutational studies are required to understand the role of membrane composition, cell wall and exo-polysaccharide role in high pH tolerance. *B. okhensis* harbors an evolved genome, well prepared to survive sudden shifts in NaCl concentration with a significantly higher number of genes involved in biosynthesis and transport of osmolytes in comparison with its non-halophilic neutrophilic relative *B. subtilis* (Table 4). Our analysis reveals that irrespective of their pH-environment, these bacterial species maintain their cytoplasmic pH close to 7 to 7.5 (Fig. 4A&B). Strikingly, *B. okhensis* genome has evolved in such a way that the membrane bound, flagella, spore formation related and other proteins being exposed constantly to external high alkaline environment contained higher acidic to basic amino acid ratio with lower pI values (Table S2, Fig 4C). Evolution of *B. okhensis* to its natural habitat, a saltpan was further confirmed by its tolerance to salt shift from 0.5% to 12% of NaCl (Fig 6A). Sudden shift from low salt to high salt led to elongated cell phenotype and one-hour lag phase but neither the growth nor the viability was influenced. Interestingly, the *B. okhensis* cell envelope is unstable in low salt, cells lose viability with time. This was observed as a decrease in cell density over time (Fig 5-●- & Fig 1-●-) indicating requirement of NaCl for stable cell density and supports its halophilic nature. The presence of Na^+ dependent transportation and presence of more predicted candidate proteins involved in sodium cycle (Table 4) act as an evidence for the genetic makeup for survival at high Na^+. Despite the presence of such sodium cycle and strong Na^+/H^+ antiporters, cells of *B. okhensis* couldn't tolerate a sudden shift in external pH from pH 7 to pH 11 (Fig. 7). We also demonstrated that the pressure created by high pH physically damaged the cells and significant proportion of them were dead with porous membrane (Fig. S2, Fig 7D). It clearly indicates that cells which can tolerate higher Na^+ in the medium need not tolerate increased OH^- in real molar concentration i.e in salt shift (0.5% to 12%), increase in NaCl/ Na^+ is ~ 2M and in pH shift from pH 7 to 11 theoretical increase in Na^+ and OH^- is only 1mM. More interestingly EDX analysis indicated Na^+ were not accumulated in the cells due to high pH shift (Fig. 8). Similar results were observed with salt shift, clearly indicating the damage to the cell wall and loss of cell viability during high pH shift is due to OH^- ion and charge imbalance (Fig. 8, Fig. 7D & Fig. S2).

Conclusions

B. okhensis is indeed a moderate halophile and an alkaliphile with optimal growth conditions of pH 10 and 5% NaCl. Our comparative genome analysis and physiological studies have shown *B. okhensis* uses acidification of the external medium and pH dependent cell wall reinforcement to survive alkaline environments. It can survive a sudden shift in NaCl concentration from 0.5% to 12% with elongated cell phenotype. In contrast, a shift from pH 7 to pH 11 has severe effect on cell growth and viability. Our observations have shown that during high pH there is hydroxyl ion induced damage on cell wall and membrane. The genome of *B. okhensis* has a relatively high number of genes involved in the metabolism of osmolytes and sodium dependent transporters in comparison to *B. subtilis.* Ectoine biosynthesis is

observed only in alkaliphilic *Bacilli* but not in *B. subtilis,* explaining the adaptation of these alkaliphiles to high salt and sodic environments. Growth of *B. okhensis* is Na^+ dependent, with a minimum requirement of 4% NaCl at neutral pH but 0.5% NaCl at pH 10 due to Na^+ dependent transport of essential nutrients. *B. okhensis* Genome encodes for a high proportion of acidic amino acids and majority of proteins have low pI values in comparison to their orthologues of *B. Subtilis.* This is a direct evidence of adaptive evolution of organism to sodic/halo-alkaline environments. Genome sequence of *B. okhensis* has also opened a new direction of considering its industrial and biotechnological importance. Although the initial interest arose from the halo-alkali tolerance capacity of this organism, this study has revealed that the genetic content of *B. okhensis* and the other alkaliphilic *Bacilli* species have majorly evolved from the sodic environments and for sodium toxicity. Further to differentiate the sodic/Na^+ stress from that of high pH/OH^- stress and to understand the evolution of alkaliphiles, extensive genome wide comparison studies are needed to delineate salt stress and alkaline stress in halophiles, non-halotolerant alkaliphiles and halo-alkaliphiles.

Methods

Strain and culture conditions

Bacillus okhensis Kh10-101^T was obtained from Japan Collection of Microorganisms (JCM) Tsukuba, Japan. Growth was monitored in CMB medium [23] with slight modifications, such as adjustment of medium pH with 10 M NaOH instead of Na_2CO_3 after sterilization. For growth measurements cells were grown in 25 ml CMB medium in 100ml culture flasks with side arms. An actively growing starter culture grown at pH 9 and 5% NaCl was used to inoculate experimental cultures. Cell density via turbidity was measured in a calorimeter with the filter set to 520 nm. pH of the medium was measured by centrifuging 50 ml of cells at respective time point at 8000 × g for 2 min at room temperature and the supernatant was used to measure the pH of the medium. Glucose estimation was done by colorimetric method [37]. For pH reduction using non-fermentable sugar, cells were grown in mineral medium with either glucose (1%) or with malate (1%) as carbon source with initial pH of 9 at the time of inoculation.

Low pH to high pH shift and low salt to high salt shift

Cells were acclimatized to pH 7 and 5% NaCl on CMB agar plates for three subcultures. Cells collected from third subculture was inoculated in to 25 ml CMB liquid medium (pH 7 and 5% NaCl concentration). Mid logarithmic phase culture was used to further inoculate into CMB medium with pH 7 and 5% NaCl. To give high pH shock (pH 11), 10M NaOH solution was added to early log phase culture. Cell viability after 30 min of high pH shock was verified using SYTOX green as per Krishna et al.,[38]. Cells are not viable upon repeated subculture at 0.5% hence for salt shift experiment, log phase cells growing in pH 10 and 0.5% NaCl were used as starter culture and inoculated into pH 10 and 0.5% NaCl medium and at designated point of time (2 h), cells were added to a flask with autoclaved NaCl so that the final concentration of NaCl is 12% in the external medium.

SEM microscopy

One milliliter of cells in the designated time point were pelleted (12000 x g, for 1 at 4^{o}C) and cell pellet was re-suspended in 1 ml of Karnovsky's fixative (5% glutaraldehyde, 4% paraformaldehyde in 0.08 M sodium phosphate (pH 7.2) buffer and incubated on ice for 30 min. Cells were then harvested (12000 x g, for 1 at 4^{o}C), and cell pellet was re-suspended in 1ml fresh Karnovsky's fixative and stored at 4 ^{o}C. For scanning electron microscope (SEM) imaging, the water in the cells was replaced with ethanol using gradient washes, from 10% to 100% with 10% increase in ethanol each round. Water replaced cells were gold sputtered and studied under scanning electron microscope (SEM). For cell length measurements, a minimum of 20 randomly selected cells were measured and average is represented as mean ± SD.

SEM-EDX measurements, 1 ml of culture was used to pellet the cells (12000 x g, for 1 minute at 4^{o}C) and the pellet was washed with 1ml of milliQ water by quickly re-suspending and centrifuging at 12000 x g, for 1 minute at 4^{o}C. The washed cell pellet was spread on stubs designated for EDX analysis, a unit area on cell was examined for Na, Mg, P, S, Cl, K, Ca, Mn, Fe, Co, Cu, Zn, elements; total signal was calculated to 100% and relative quantity of individual ions was measured.

16S rDNA phylogenetic analysis

Bacillus spp with reported whole genome sequences (Jan 2016) were selected for phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [39]. The bootstrap consensus tree inferred from 500 replicates [40] is taken to represent the evolutionary history of the taxa analyzed [41]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [40]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 1011 positions in the final dataset. Evolutionary analyses were conducted in MEGA7[41].

Genome annotation and comparative analysis

Annotation was done using RAST tool and the number of representative genes coding for each subsystem were represented as per the RAST seed subsystems [42]. For comparison of genomes, genome sequences of *B. okhensis* Kh10-101^{T} (GCA_000787375.1), *B. pseudofirmus* OF4 (GCA_000005825.2), *B. halodurans* C-125 (GCA_000011145.1), *B. alcalophilus* ATCC 27647 (GCA_000009825.1), *B. clausii* KSM-K16 (GCA_000292245.2) and *B. subtilis* 168 (GCA_000009045.1) were downloaded from NCBI and submitted for RAST annotation and genes encoding corresponding proteins were searched as per the functional annotation. Identified subsystem features numbers were indicated in tables and corresponding protein id from NCBI was presented in text. All the predicted

proteins from the RAST annotated, predicted proteomes were submitted to online tools http://web.expasy.org/cgi-bin/compute_pi/pi_tool for pI calculation.

Identification of bidirectionally best hits and comparison of their pI values with acidic to basic amino acid composition:

To find the orthologs between *B. okhensis* and *B. subtilis* the protein databases were created with the stand alone version of **BlastP** downloaded from the NCBI website ("ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/"). The fasta format file was generated for both *B. okhensis* and *B. subtilis*. These files having the protein sequences in fasta format were given as input and protein databases of both *Bacillus* species were created in Blast+. For identifying the true orthologs by bidirectionally best-hit method all the proteins of *B. okhensis* taken as query sequences were subjected to Blastp against the *B. subtiius* protein database as target. Similarly, *Bacillus subtilis* proteins were taken as query sequences and *B. okhensis* protein data base as target. The percentage identity, E-value and the bit score were taken as parameters to choose the best hit. The normalized frequencies of all 20 amino acids of each and every protein were calculated using RStudio and the pI values were calculated. For every protein, acidic to basic amino acids' ratio (D+E/K+R+H) was calculated by adding the acidic residues and dividing it by the sum of the basic residues and a graph was plotted between pI values of every orthologous proteins and their corresponding Acid to base ratios.

Abbreviations

NaCl:sodium chloride; NaOH:sodium hydroxide; Na^+:sodium ion; OH^-:hydroxyl ion; SEM:scanning electron microscopy; EDX:Energy-dispersive X-ray spectroscopy; SD:standard deviation; D:aspartic acid; E:glutamic acid; H:histidine; K:lysine; R:arginine; G: glycine; A:alanine; S:serine; V:valine; I:iso-leucine; N:aspargine; RAST: Rapid Annotations using Subsystems Technology; pI:iso electric point; CMB:nutrient medium (1% D-glucose, 0.5% KH_2PO_4, 0.5% peptone, 0.5% yeast extract); NhaA-D: sodium/hydrogen antiporters A-D; Kef: potassium efflux protein; Ktr: potassium transporter; MFS: Major Facilitator Super family protein; MurJ: Murein biosynthesis integral membrane protein; YfcH: cell division inhibitor protein; Mot complex: flagellar motor protein complex

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available in manuscript and in supplementary information. All data generated or analysed during this study are also available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by a grant from UGC-ISF research project 6-6/2017(IC) and DBT funded project (BT/PR12661/NDB/39/518/2015) to J.S.S.P.

Acknowledgements

We thank Dr. Catriona MA Thompson, Department of Molecular Microbiology, John Innes Center, Norwich, UK for critical reading of the manuscript. We gratefully acknowledge the Department of Science and Technology, Government of India, Funds for Infrastructure in Science and Technology, Level I and UGC-Special Assistance Programme support to the Department of Biotechnology and Bioinformatics. We thank Department of Biotechnology, Govt. of India, Centre for Research and Education in Biology and Biotechnology for providing Genomics facility and support of DST-Promotion of University Research and Scientific Excellence to the School of Life Sciences, University of Hyderabad.

Authors' contributions

PSK, JSSP designed and coordinated the study; PSK, JSSP: wrote the manuscript; PSK, SR, JSSP: performed bioinformatics analyses and edited the manuscript; PSK: carried out laboratory work. All authors have contributed and approved the final manuscript.

References

1. Krulwich TA, Sachs G, Padan E: **Molecular aspects of bacterial pH sensing and homeostasis**. *Nat Rev Microbiol* 2011, **9**(5):330-343.
2. Padan E, Bibi E, Ito M, Krulwich TA: **Alkaline pH homeostasis in bacteria: New insights**. *Bba-Biomembranes* 2005, **1717**(2):67-88.
3. Preiss L, Hicks DB, Suzuki S, Meier T, Krulwich TA: **Alkaliphilic Bacteria with Impact on Industrial Applications, Concepts of Early Life Forms, and Bioenergetics of ATP Synthesis**. *Front Bioeng Biotechnol* 2015, **3**:75.
4. Horikoshi K: **Alkaliphiles: Some applications of their products for biotechnology**. *Microbiol Mol Biol R* 1999, **63**(4):735-+.
5. Krulwich TA, Guffanti AA: **Alkalophilic Bacteria**. *Annu Rev Microbiol* 1989, **43**:435-463.
6. Yumoto I: **Bioenergetics of alkaliphilic Bacillus spp.** *J Biosci Bioeng* 2002, **93**(4):342-353.

7. Sarethy IP, Saxena Y, Kapoor A, Sharma M, Sharma SK, Gupta V, Gupta S: **Alkaliphilic bacteria: applications in industrial biotechnology**. *J Ind Microbiol Biot* 2011, **38**(7):769-790.
8. Hicks DB, Liu J, Fujisawa M, Krulwich TA: **F1F0-ATP synthases of alkaliphilic bacteria: Lessons from their adaptations**. *Bba-Bioenergetics* 2010, **1797**(8):1362-1377.
9. Kitada M, Kosono S, Kudo T: **The Na+/H+ antiporter of alkaliphilic Bacillus sp.** *Extremophiles : life under extreme conditions* 2000, **4**(5):253-258.
10. Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, Fuji F, Hirama C, Nakamura Y, Ogasawara N *et al*: **Complete genome sequence of the alkaliphilic bacterium Bacillus halodurans and genomic sequence comparison with Bacillus subtilis**. *Nucleic Acids Res* 2000, **28**(21):4317-4331.
11. Janto B, Ahmed A, Ito M, Liu J, Hicks DB, Pagni S, Fackelmayer OJ, Smith TA, Earl J, Elbourne LDH *et al*: **Genome of alkaliphilic Bacillus pseudofirmus OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4**. *Environ Microbiol* 2011, **13**(12):3289-3309.
12. Takami H, Takaki Y, Uchiyama I: **Genome sequence of Oceanobacillus iheyensis isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments**. *Nucleic Acids Res* 2002, **30**(18):3927-3935.
13. Aono R, Ito M, Joblin KN, Horikoshi K: **A High Cell-Wall Negative Charge Is Necessary for the Growth of the Alkaliphile Bacillus-Lentus C-125 at Elevated Ph**. *Microbiol-Uk* 1995, **141**:2955-2964.
14. Aono R, Ito M, Machida T: **Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile Bacillus lentus C-125**. *J Bacteriol* 1999, **181**(21):6600-6606.
15. Fujisawa M, Fackelmayer OJ, Liu J, Krulwich TA, Hicks DB: **The ATP Synthase a-subunit of Extreme Alkaliphiles Is a Distinct Variant MUTATIONS IN THE CRITICAL ALKALIPHILE-SPECIFIC RESIDUE LYS-180 AND OTHER RESIDUES THAT SUPPORT ALKALIPHILE OXIDATIVE PHOSPHORYLATION**. *J Biol Chem* 2010, **285**(42):32105-32115.
16. Ivey DM, Krulwich TA: **Two unrelated alkaliphilic Bacillus species possess identical deviations in sequence from those of other prokaryotes in regions of F0 proposed to be involved in proton translocation through the ATP synthase**. *Res Microbiol* 1992, **143**(5):467-470.
17. Liu J, Fujisawa M, Hicks DB, Krulwich TA: **Characterization of the Functionally Critical AXAXAXA and PXXEXXP Motifs of the ATP Synthase c-Subunit from an Alkaliphilic Bacillus**. *J Biol Chem* 2009, **284**(13):8714-8725.
18. Wang ZX, Hicks DB, Guffanti AA, Baldwin K, Krulwich TA: **Replacement of amino acid sequence features of a- and c-subunits of ATP synthases of alkaliphilic Bacillus with the Bacillus consensus sequence results in defective oxidative phosphorylation and non-fermentative growth at pH 10.5**. *J Biol Chem* 2004, **279**(25):26546-26554.
19. Hicks DB, Krulwich TA: **The Respiratory-Chain of Alkaliphilic Bacteria**. *Bba-Bioenergetics* 1995, **1229**(3):303-314.

20. Krulwich TA, Ito M, Guffanti AA: **The Na+-dependence of alkaliphily in Bacillus**. *Bba-Bioenergetics* 2001, **1505**(1):158-168.
21. Krulwich TA, Hicks DB, Ito M: **Cation/proton antiporter complements of bacteria: why so large and diverse?** *Mol Microbiol* 2009, **74**(2):257-260.
22. Krulwich TA, Ito M, Gilmour R, Guffanti AA: **Mechanisms of cytoplasmic pH regulation in alkaliphilic strains of Bacillus**. *Extremophiles* 1997, **1**(4):163-169.
23. Nowlan B, Dodia MS, Singh SP, Patel BKC: **Bacillus okhensis sp nov., a halotolerant and alkalitolerant bacterium from an Indian saltpan**. *Int J Syst Evol Micr* 2006, **56**:1073-1077.
24. Krishna PS, Sreenivas A, Singh DK, Shivaji S, Prakash JS: **Draft genome sequence of Bacillus okhensis Kh10-101T, a halo-alkali tolerant bacterium from Indian saltpan**. *Genom Data* 2015, **6**:283-284.
25. Wolfe AJ: **The acetate switch**. *Microbiol Mol Biol R* 2005, **69**(1):12-+.
26. Aono R, Ohtani M: **Loss of Alkalophily in Cell-Wall-Component-Defective Mutants Derived from Alkalophilic Bacillus C-125 - Isolation and Partial Characterization of the Mutants**. *Biochem J* 1990, **266**(3):933-936.
27. Clejan S, Krulwich TA, Mondrus KR, Setoyoung D: **Membrane Lipid-Composition of Obligately and Facultatively Alkalophilic Strains of Bacillus Spp**. *J Bacteriol* 1986, **168**(1):334-340.
28. Dunkley EA, Guffanti AA, Clejan S, Krulwich TA: **Facultative Alkaliphiles Lack Fatty-Acid Desaturase Activity and Lose the Ability to Grow at near-Neutral Ph When Supplemented with an Unsaturated Fatty-Acid**. *J Bacteriol* 1991, **173**(3):1331-1334.
29. Sleator RD, Hill C: **Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence**. *FEMS microbiology reviews* 2002, **26**(1):49-71.
30. Oren A, Heldal M, Norland S, Galinski EA: **Intracellular ion and organic solute concentrations of the extremely halophilic bacterium Salinibacter ruber**. *Extremophiles* 2002, **6**(6):491-498.
31. Oren A: **Life at high salt concentrations, intracellular KCl concentrations, and acidic proteomes**. *Front Microbiol* 2013, **4**.
32. Ito M, Xu HX, Guffanti AA, Wei Y, Zvi L, Clapham DE, Krulwich TA: **The voltage-gated Na+ channel NavBP has a role in motility, chemotaxis, and pH homeostasis of an alkaliphilic Bacillus**. *P Natl Acad Sci USA* 2004, **101**(29):10566-10571.
33. Smith TJ, Blackman SA, Foster SJ: **Autolysins of Bacillus subtilis: multiple enzymes with multiple functions**. *Microbiol-Uk* 2000, **146**:249-262.
34. den Besten HMW, Mols M, Moezelaar R, Zwietering MH, Abee T: **Phenotypic and Transcriptomic Analyses of Mildly and Severely Salt-Stressed Bacillus cereus ATCC 14579 Cells**. *Appl Environ Microb* 2009, **75**(12):4111-411
35. Wiegert T, Homuth G, Versteeg S, Schumann W: **Alkaline shock induces the Bacillus subtilis sigma(W) regulon**. *Molecular microbiology* 2001, **41**(1):59-71.

36. Oren A: **Industrial and environmental applications of halophilic microorganisms.** Environ Technol 2010, 31(8): 825-34.

37. Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F: **A Colorimetric Method for the Determination of Sugars**. *Nature* 1951, **168**(4265):167-167.

38. Krishna PS, Rani BR, Mohan MK, Suzuki L, Shivaji S, Prakash JSS: **A novel transcriptional regulator, Sll1130, negatively regulates heat-responsive genes in Synechocystis sp PCC6803**. *Biochem J* 2013, **449**:751-760.41.

39. Tamura K, Nei M: **Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial-DNA in Humans and Chimpanzees**. *Mol Biol Evol* 1993, **10**(3):512-526.

40. Felsenstein J: **Confidence-Limits on Phylogenies - an Approach Using the Bootstrap**. *Evolution* 1985, **39**(4):783-791.

41. Kumar S, Stecher G, Tamura K: **MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets**. *Mol Biol Evol* 2016, **33**(7):1870-1874.

42. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M *et al*: **The RAST Server: rapid annotations using subsystems technology**. *BMC Genomics* 2008, **9**:75.

Tables

Table 1. General features of *B. okhensis* genome in comparison with other *Bacilli* species

Feature	Kh10-101^T	OF4	C-125	ATCC 27647	KSM-K16	168
Accession No. (GCA_)	000787375.1	000005825.2	000011145.1	000292245.2	000009825.1	000009045.1
chromosome size (bp)	4,865,284 bp	3,858,997 bp	4,202,352 bp	4,370,295 bp	4,303,871	4,215,606 bp
G+C content (mol %)	38.2	39.9	43.7	37.2	44.7	43.7
Coding sequences (CDS)	4952	3890	4102	4328	4099	4114
Percentage of coding region	84.6	86.4	85	81	86.7	87
Start codon %						
ATG	78	81	78	78	75	78
GTG	11	9	12	12	14	12
TTG	11	10	10	10	11	10
RNA coding genes	165	97	163	86	150	181
F_1F_0-ATP synthase c-subunit N-terminal motif	GGSIAVA	AGAIAVA	GGAIAVA	GGAIAVA	GGAIGVA	GAGIGNG
Growth range (pH)	7 - 11	7.5 -11.4	7 - 11	8.5-11.5	7-10.5	6 – 9
Growth range (NaCl)	Up to 12 %	Up to 15%	Up to 10%	Up to 8%	Up to 10%	Up to 6%

Features of the *B. okhensis* genome and comparison with the genomes of other *Bacillus* species. *B. okhensis* Kh10-101^T(Kh10-101^T), *B. pseudofirumus* OF4 (OF4), *B. halodurans* C-125 (C-125), *B. alcalophilus* ATCC 27647 (ATCC 27647), *B. clausii* KSM-K16 (KSM-K16) and *B. subtilis* 168 (168).

Table 2. RAST subsystem features for organic acids metabolism and fermentation

RAST subsystem feature	Kh10-101^T	OF4	C-125	ATCC 27647	KSM-K16	168
Organic acids metabolism						
CitAB	ND	ND	ND	2	2	2
Propionate-CoA to succinate module	ND	ND	5	5	5	ND
Methylcitrate cycle	ND	ND	4	5	6	3
Glycerate metabolism	9	7	6	6	6	8
Lactate utilization	4	5	8	6	5	6
Citrate Metabolism, Transport, and Regulation	ND	ND	ND	ND	ND	5
Alpha-acetolactate operon	ND	ND	ND	ND	ND	2
Malonate decarboxylase	ND	9	ND	ND	ND	ND
Fermentation						
Fermentation to mixed acids	12	ND	ND	12	ND	ND
Acetolactate synthase sub units	2	2	4	2	3	2
Fermentation to lactate	5	3	3	3	3	3
Fermentation to butyrate	19	21	21	17	9	14
Acetoin, butane diol metabolism	3	ND	14	ND	9	10

Number of candidate genes involved in metabolism of organic acids and fermentation to organic acids in *B. okhensis* genome and comparison with the genomes of other *Bacillus* species. *B. okhensis* Kh10-101^T(Kh10-101^T), *B. pseudofirumus* OF4 (OF4), *B. halodurans* C-125 (C-125), *B. alcalophilus* ATCC 27647 (ATCC 27647), *B. clausii* KSM-K16 (KSM-K16), and *B. subtilis* 168 (168). No RAST subsystem feature detected (ND).

Table 3. RAST subsystem features for cell wall and capsule synthesis

Feature - cell wall and capsule synthesis	Kh10-101^T	OF4	C-125	ATCC 27647	KSM-K16	168
Gram stain	+ ve	+ ve	+ ve	+ ve	+ ve	+ve
Capsular and extracellular polysaccharides						
dTDP-rhamnose synthesis	ND	ND	4	ND	4	5
CMP-N-acetylneuraminate Biosynthesis	6	ND	ND	ND	ND	3
Polysaccharide deacetylases	6	6	3	5	ND	4
Rhamnose containing glycans	ND	ND	11	ND	10	12
Exopolysaccharide Biosynthesis	5	5	7	8	8	7
Sialic Acid Metabolism	14	13	ND	12	24	18
Cell wall and capsule						
Murein Hydrolases	20	10	13	13	15	14
Peptidoglycan Biosynthesis	ND	31	30	34	31	29
UDP-N-acetylmuramate from Fructose-6-phosphate	6	7	9	7	10	7
Biosynthesis	2	2	2	2	4	3
YjeE	11	4	8	5	8	8
Recycling of peptidoglycan amino acids	ND	ND	ND	1	ND	ND
Recycling of peptidoglycan amino sugars	7	5	5	7	6	5
Peptidoglycan biosynthesis--gjo						
Gram positive cell wall components						
Teichoic and lipoteichoic acids biosynthesis	7	3	4	9	8	19
D-Alanyl Lipoteichoic Acid Biosynthesis	ND	ND	ND	ND	ND	4
Teichuronic acid biosynthesis	ND	ND	5	ND	2	7
Sortase	ND	ND	6	ND	4	1
Polyglycerolphosphatelipoteichoic acid biosynthesis	2	ND	ND	1	6	4
Gram negative cell wall components						
Peptidoglycan lipid II flippase	2	ND	ND	ND	ND	ND

Number of RAST subsystem feature counts for cell wall and capsule synthesis in *B. okhensis* genome and comparison with the genomes of other *Bacillus* species. *B. okhensis* Kh10-101^T(Kh10-101^T), *B. pseudofirumus* OF4 (OF4), *B. halodurans* C-125 (C-125), *B. alcalophilus* ATCC 27647 (ATCC 27647), *B. clausii*KSM-K16 (KSM-K16), and *B. subtilis* 168 (168). No subsystem feature by RAST (ND).

Table 4. Candidate genes for sodium antiporters, sodium symporters and sodium dependent transport

S. No	Feature	Kh10-101^T	OF4	C-125	ATCC 27647	KSM-K16	168
1	**Sodium antiporters**						
(a)	NhaA	ND	ND	ND	ND	ND	ND
(b)	NhaB	ND	ND	ND	ND	ND	ND
(c)	NhaC	1	3	1	1	2	1
(d)	NhaD	1	1	ND	ND	ND	ND
(e)	Na+/H+ antiporter subunits	12	12	11	17	16	10
(f)	Na+/drug antiporter	4	4	4	2	2	1
2	Sodium symporter	8	10	10	4	10	9
3	Sodium transporter	10	11	9	8	7	4
4	Ectoine synthase	1	2	1	1	2	ND

Candidate genes involved in sodium dependent transport in *B. okhensis* genome and comparison with the genomes of other *Bacillus* species. *B. okhensis* Kh10-101^T(Kh10-101^T), *B. pseudofirumus* OF4 (OF4), *B. halodurans* C-125 (C-125), *B. alcalophilus* ATCC 27647 (ATCC 27647), *B. clausii*KSM-K16 (KSM-K16), and *B. subtilis* 168 (168). No homologue was detected (ND).

Figures

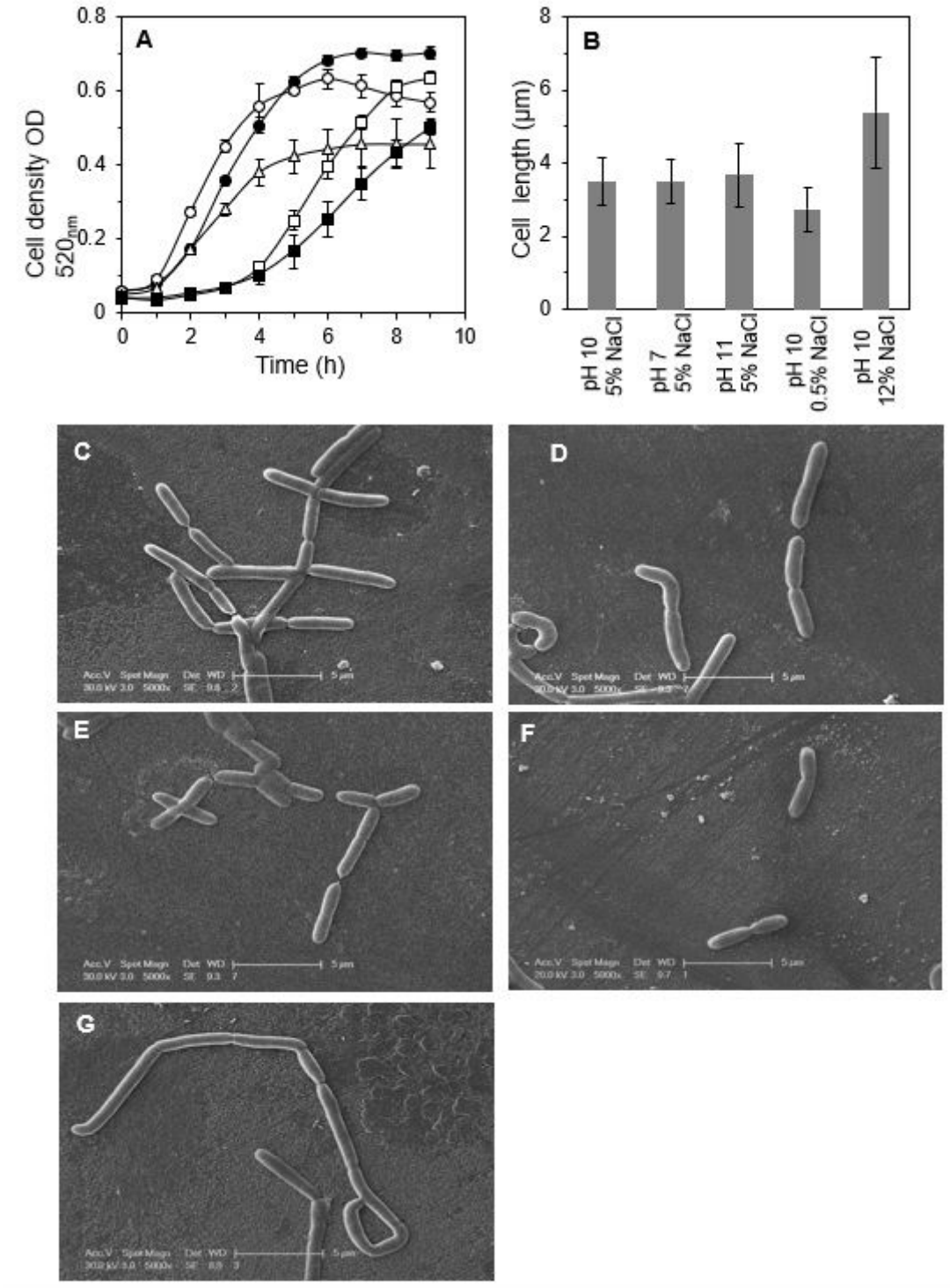

Figure 1

Growth profiles of Bacillus okhensis in different combinations of pH and salt grown at 37 ◦C, 180 rpm (A); pH 10 and 5% NaCl (-●-), pH 10 and 0.5% NaCl (-●-), pH 7 and 5% NaCl (-Δ-), pH 10 and 12% NaCl (-■-), pH 11 and 5% NaCl (-□-). Similar results were obtained in three independent experiments, and the data represented as means ± SD. Cell morphology, cell size of B. okhensis at different conditions; (B) Average cell length in each condition is shown, cell size for a minimum of 20 cells were measured from Scanning electron microscopy (SEM) images and the data are represented as means ± SD. SEM images of B.

okhensis grown at different combinations of pH and NaCl: pH 10 and 5% NaCl (C), pH 7 and 5% NaCl (D), pH 11 and 5% NaCl (E), pH 10 and 0.5% NaCl (F), pH 10 and 12% NaCl (G). Scale bar = 5 μm.

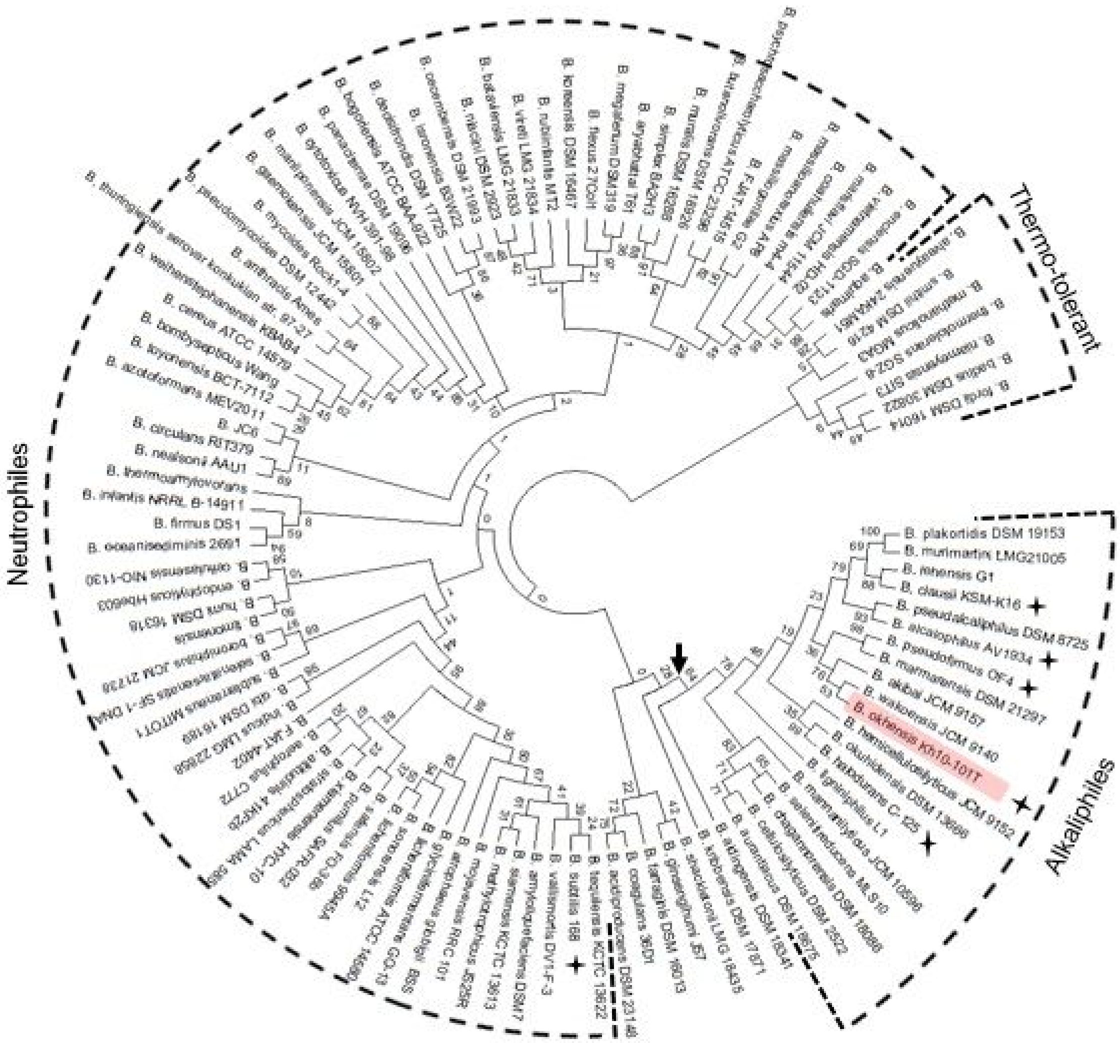

Figure 2

16S based phylogenetic relation of B. okhensis with other Bacillus species; neighbor joining phylogenetic dendrogram on a comparison of the 16S rRNA gene sequence of B. okhensis and all Bacillus species with complete genome sequences. B. okhensis was highlighted by a colored rectangle. Probable ancestor of alkaliphilic Bacilli is indicated by an arrow in figure. Asterisk indicates species that were used for comparison.

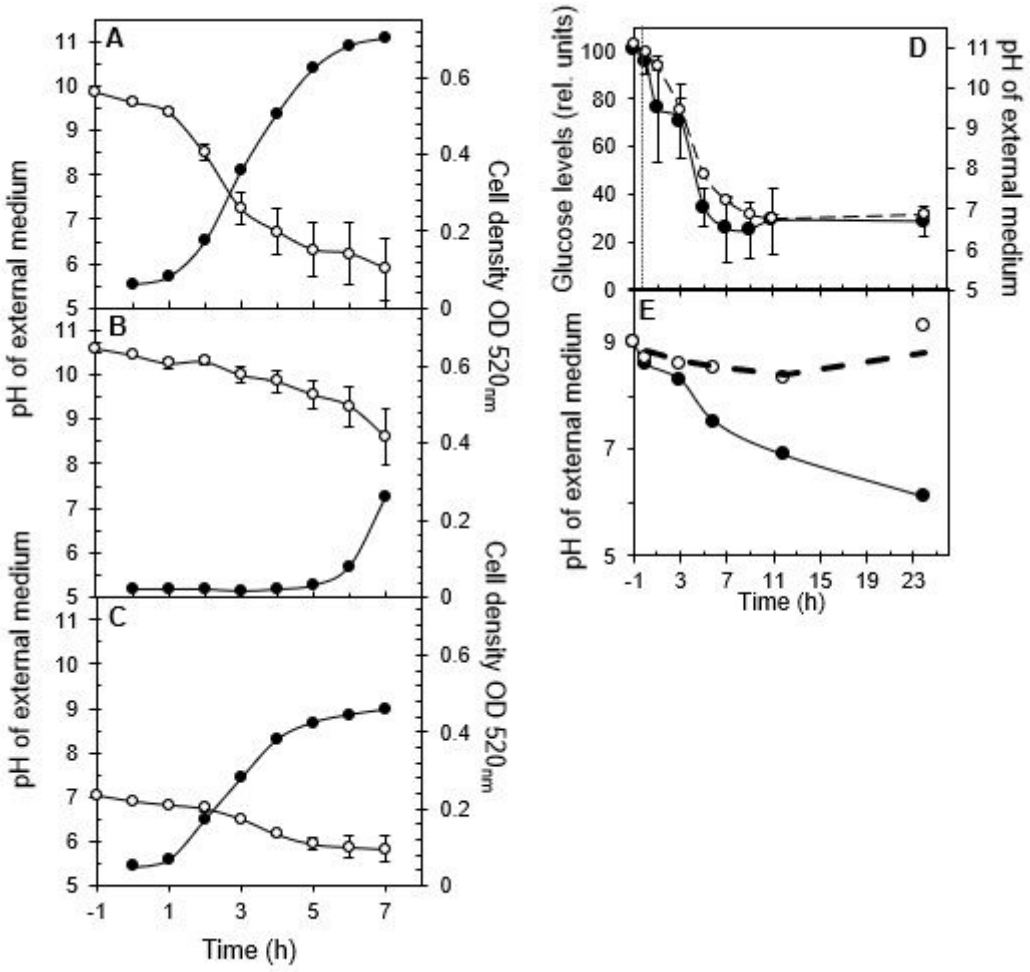

Figure 3

Reduction of external pH as an adaptation method; the pH of the external medium and cell density that were inoculated (pH 9 and 5% starter culture) into different combinations of initial pH and NaCl. pH 10 and 5% NaCl (A), pH 11 and 5% NaCl pH 7 and 5% NaCl (B), pH 7 and 5% NaCl (C), growth and pH of external medium was measured in each condition. Similar results were obtained in three independent experiments and the data represented as means ± SD. Consumption of glucose from the external medium and corresponding pH (D); Glucose concentration in the external medium (-●-), pH of the external medium (-●-). Cells adapted to pH 11 for 3 subcultures were used as the initial inoculum, decrease in glucose concentration and pH of the medium were monitored. Similar results were obtained in three independent experiments, and the data represented as means ± SD. Changes in the external medium pH in minimal medium (E). Fermentable carbon source glucose (-●-) and non-fermentable carbon source malate (-●-), similar results were obtained in two individual experiments and data represented is the mean.

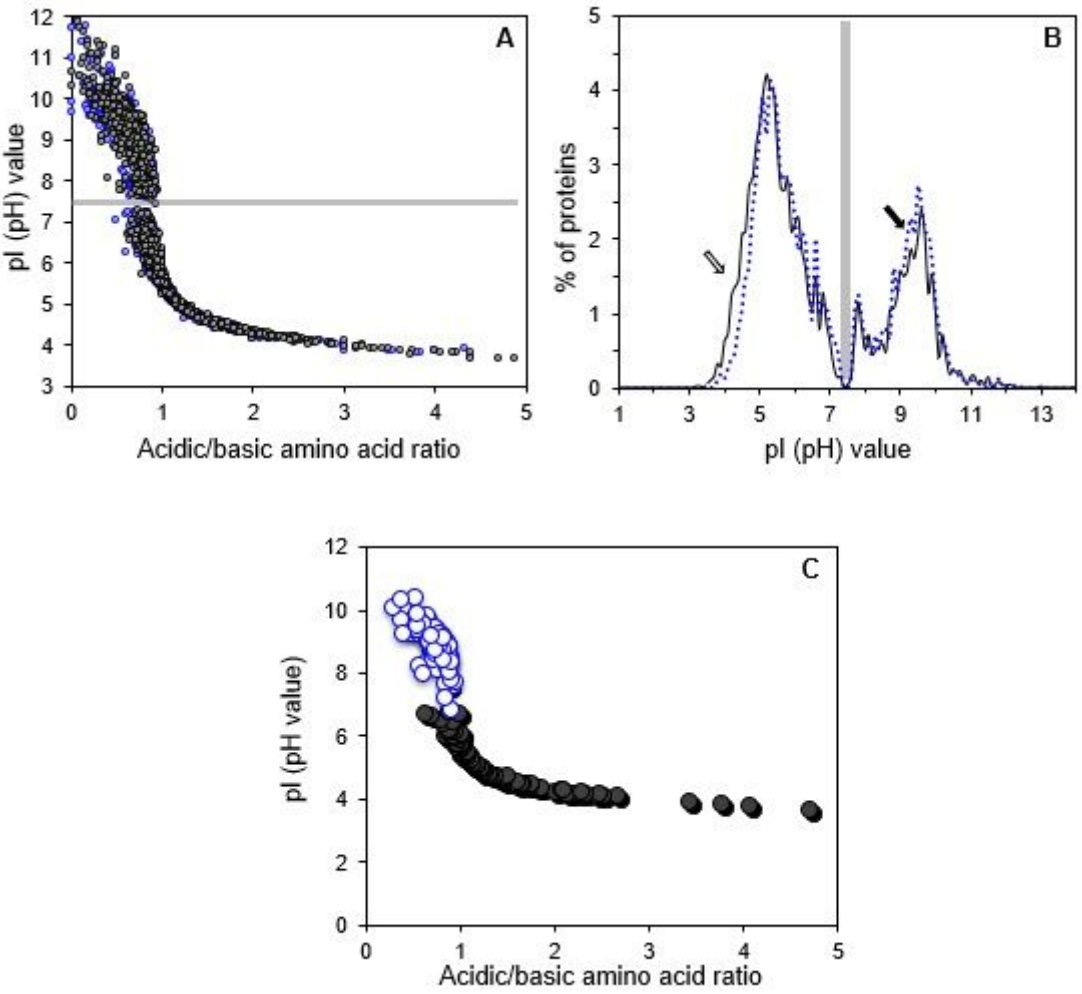

Figure 4

Comparison of acidic/basic amino acid ratio and pI of halo-alkalophilic B. okhensis proteins with that of B. subtilis. (A) The acidic/basic amino acid ratio of every protein is plotted against the pI value of the corresponding protein, B. okhensis (black); B. subtilis (blue). Probable cytoplasmic pH highlighted grey in figure. Calculated pI frequency of the proteome (B); B. okhensis Kh10-101T (black), and B. subtilis 168 (blue dotted line). Probable cytoplasmic pH highlighted grey in figure. Drift in pI frequency towards acidic and alkaline pH is indicated with open and closed arrows respectively. (C) B. okhensis proteins with relatively higher acidic to basic aminoacid ratio and low pI values were compared with that of their orthologs from B. subtilis. pI values of proteins were plotted against their acidic/basic amino acid ratios, B. okhensis (solid circle) and B.subtilis (open blue circle). List of orthologous proteins with significant differences in their pI values are listed in Table S2.

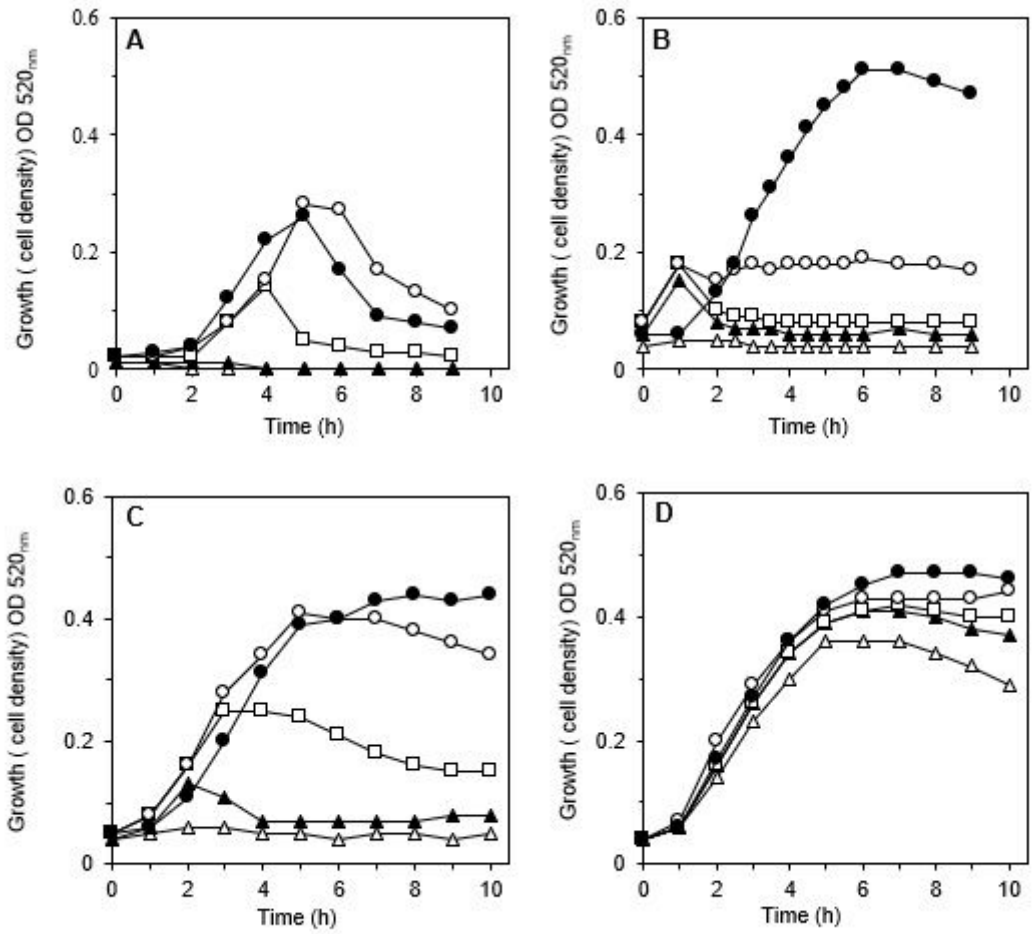

Figure 5

Sodium dependency of growth profiles of B. okhensis in different sub-optimal salt concentrations grown at 37 ◦C, 180 rpm. 0 % NaCl. (A); 0.5 % NaCl (B); 1 % NaCl (C); and 4 % NaCl (D). in each panel pH 7 (-Δ-); pH 7.5 (-▲-); pH 8 (-□-); pH 9 (-●-) and pH 10 (-●-). Similar results were obtained in three independent experiments, and the data represented as means ± SD.

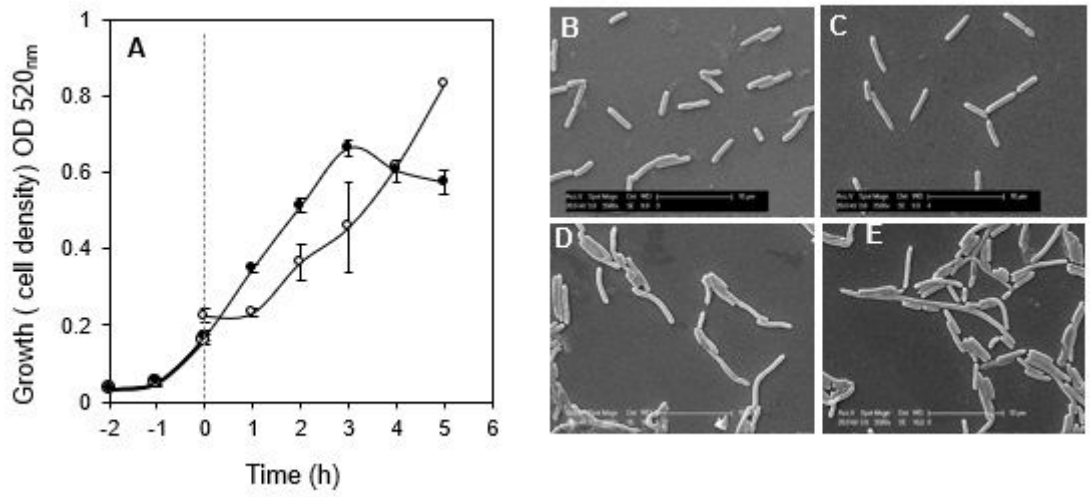

Figure 6

Growth of B. okhensis with sudden shift to high salt (A) culture growing in pH 10 and 0.5% NaCl (unshifted) (-■-) culture shifted to pH 10 and 12% NaCl (-●-). Point of addition of NaCl to attain 12% of NaCl is indicated with dotted line in the figure. Similar results were obtained in three independent experiments and the data represented as means ± SD. Effect of salt shift (0.5%-12%) in cell morphology; cells just before shift (B), 30 min after shift (C), 60 min after shift (D) and 180 min after shift (E), representative images were shown at magnification of 3500X, scale bar = 10 µm.

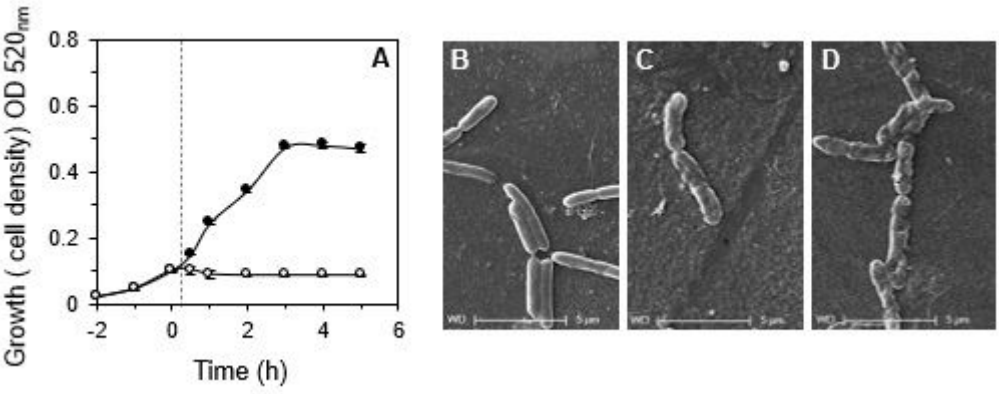

Figure 7

Growth profile of B. okhensis with a sudden shift in pH (A) culture growing in pH 7 and 5% NaCl (unshifted) (-●-) culture shifted to pH 11 and 5% NaCl (-●-); Effect of pH shift (pH 7-pH 11) on cell morphology; cells just before shift (B), 30 min after shift (C), 180 min after shift (D), representative images were shown at magnification of 5000x bar in the picture shows 5 µm. Effect of pH shift (pH 7-pH 11) on cell morphology; cells just before shift (B), 30 min after shift (C), 180 min after shift (D), representative images were shown at magnification of 5000X, scale bar = 5 µm.

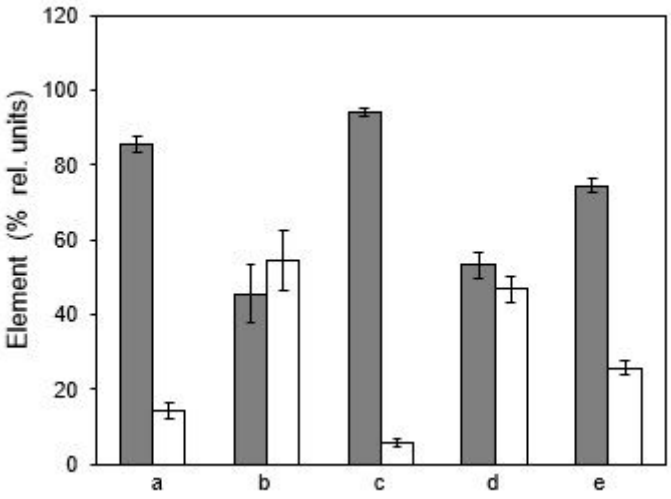

Figure 8

Amount of sodium and potassium elements in the cell in different conditions; elemental composition in cells were analyzed, sodium (grey bar), potassium (white bar). pH 10 and 5% NaCl (a), pH 7 and 5% NaCl (b), 30 min after pH7 - pH 11 shift (c), pH 10 and 0.5% NaCl (d), 30 min after 0.5%-12% NaCl (e). cellular elemental composition was measured using SEM associated EDX for (Na, Mg, P, S, Cl, K, Ca, Mn, Fe, Co, Cu, Zn) relative amounts of element concentration calculating the total to 100% was represented. Similar results were obtained in three independent experiments and the data represented as means ± SD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplmentaryfigures.pptx
- TableS2.xlsx
- TableS1Supfiglegends.docx