# Genomics of Alkaliphiles

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**Abstract**

Alkalinicity presents a challenge for life due to a "reversed" proton gradient that is unfavourable to many bioenergetic processes across the membranes of microorganisms. Despite this, many bacteria, archaea, and eukaryotes, collectively termed alkaliphiles, are adapted to life in alkaline ecosystems and are of great scientific and biotechnological due to their niche specialization and ability to produce highly stable enzymes. Advances in next-generation sequencing technologies have propelled not only the genomic characterization of many alkaliphilic microorganisms that have been isolated from nature alkaline sources, but also our understanding of the functional relationships between different taxa in microbial communities living in these ecosystems. In this review, we discuss the genetics and molecular biology of alkaliphiles from an 'omics' point of view, focusing on how metagenomics and transcriptomics have contributed to our understanding of these extremophiles.

## Introduction- Alkaliphiles in the Metagenomics era

Alkaliphiles are a diverse group of micro-organisms that are defined as being able to grow in high pH environments (≥9). These organisms reside in a range of extreme environments in which high alkalinity has been established through geological processes, such as the accumulation of $CO_2$ and subsequent production of carbonate/bicarbonate-rich solutions in soda lakes, as well as transient

biological events such as ammonification and sulfate reduction in soils (Grant, 2009). In these extreme niches, microbial communities are not only well adapted to alkalinity, but they must also cope with a range of other environmental stresses, including high salinity, low or high temperatures, and oxygen deprivation (Yumoto, 2007). Consequently, alkaliphiles are of great scientific and biotechnological interest due to their highly selective niche specialization and their ability to produce proteins that are stable across a wide range of extreme conditions (Grant et al. 1990).

Since the first report of an alkaliphile genome sequence in 2000 (Takami et al., 2000), the number of sequenced genomes of alkaliphilic microorganisms has increased exponentially due to advances in next-generation sequencing technologies (Koboldt et al. 2013). Currently, the JGI genome portal (Nordberg et al. 2014) lists the sequences of 288 genomes from isolated microorganisms that have been characterized to grow in alkaline conditions. These genomes are distributed across 10 different phyla (Figure 1A), and are highly variable in terms of GC-content (from 26% to 74%) and genome size (from 1.6Mb to 11Mb) (Figure 1B). This high genetic variability, together with the range of habitats from which alkaliphiles have been isolated, reflects the functional diversity of alkaliphilic microorganisms. As such, no obvious trends can be found between the alkaliphilic phenotype and particular genetic features. The phylum Firmicutes, which includes the historically relevant species *Bacillus halodurans* C-125 and *B. pseudofirmus* OF-4 (Takami et al. 2000; Janto et al. 2011), represents the second largest fraction of sequenced genomes (83 genomes), most of which have a GC content below 50 %. By comparison, the JGI lists 114 Proteobacteria alkaliphile genomes, the majority of which have a high GC content (≥ 50%). These include *Halomonas* sp. GFAJ-1, which is capable of thriving in arsenic-rich environments and has been associated with arsenate detoxification (Wu et al. 2018). To date, only two genomes for alkaliphilic Cyanobacteria have been listed in the JGI database, despite the fact that members of this phylum play a crucial role as primary photoautotrophic producers in many alkaline environments (Zavarzin et al. 1999). The two genomes belong to the desiccation-tolerant *Chroococcidiopsis thermalis* PCC 7203, and *Arthrospira platensis* C1, which is cultivated at large industrial scale as a food product for both humans and animals

(Cheevadhanarak et al. 2012). All 16 publicly available alkaliphilic archaeal genomes belong to the Euryarchaeota phylum, and the majority of these organisms have been isolated from highly saline fresh water environments. Genome sizes for these archaea vary between 1.8 Mb to 4.9 Mb, and 12 have a very high CG content (≥ 60%). High genomic GC content is a common feature of halophiles, and has been associated to adaptation mechanisms against UV-induced thymidine dimer formation and the consequent accumulation of mutations (Paul et al. 2008). Ten other alkaliphilic cyanobacterial genomes that are not listed in the JGI database have also been sequenced, including five additional *Arthrospira* species (Klanchui et al. 2017).

Compared to prokaryotic alkaliphiles, there is a dearth of knowledge on the genomics alkaliphilic eukaryotes. Crucially the recent drive for culture-independent techniques such as metagenomics has led to in-depth studies into the composition, functional capacities and ecological impact of communities living in alkaline environments. To date, 153 metagenomes have been obtained from alkaline environments, the majority of which were sourced from saline and alkaline water (51 metagenomes), and serpentinite rock and fluid (40 genomes). Metagenomics studies have also been crucial in detecting eukaryotic life in high pH environments. Members of the genera *Frontania* and *Lacrymaria*, both ciliates, were reported to be found in four distinct alkaline environments, while diatoms of the class Fragillariophyceae were found in both alkaline and acidic habitats (Amaral-Zettler, 2012).

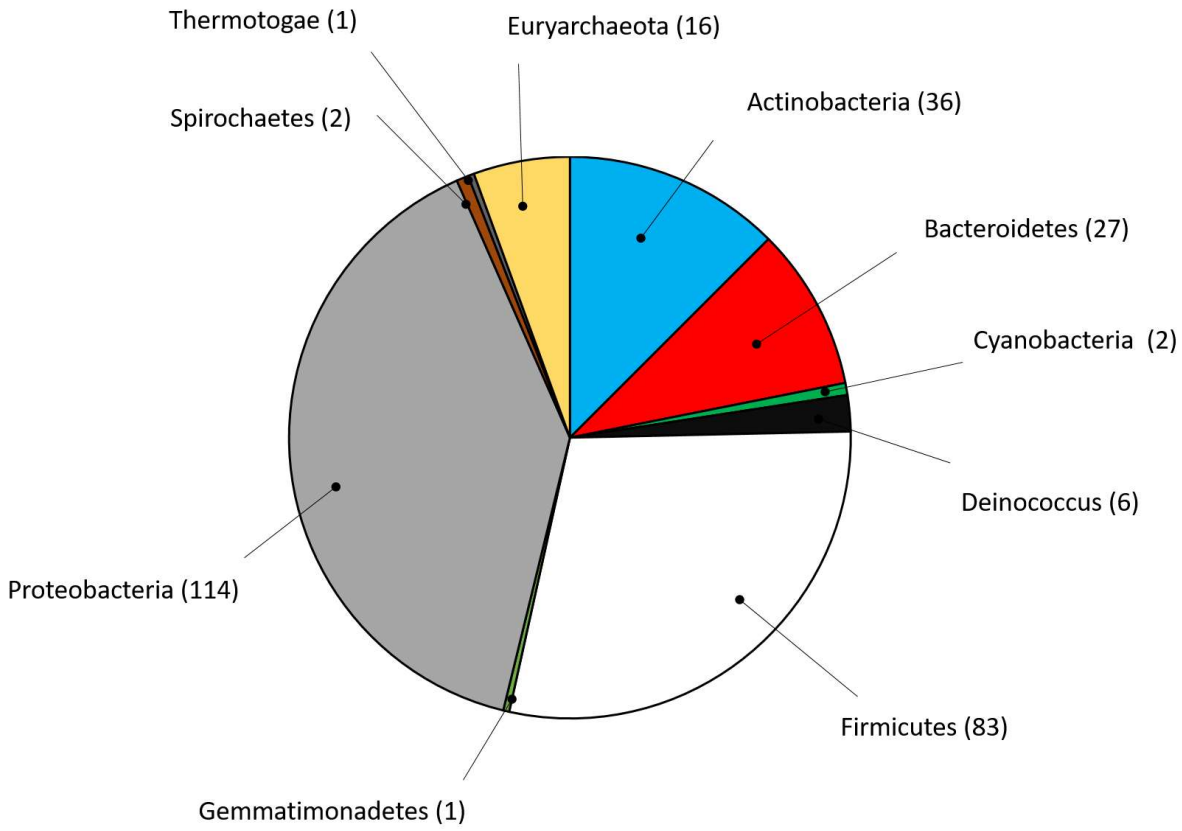


**Figure 1A** – Distribution of the JGI-listed alkaliphile genomes across different phyla. The number of genomes for each phylum is indicated in brackets next to the name for that phylum.

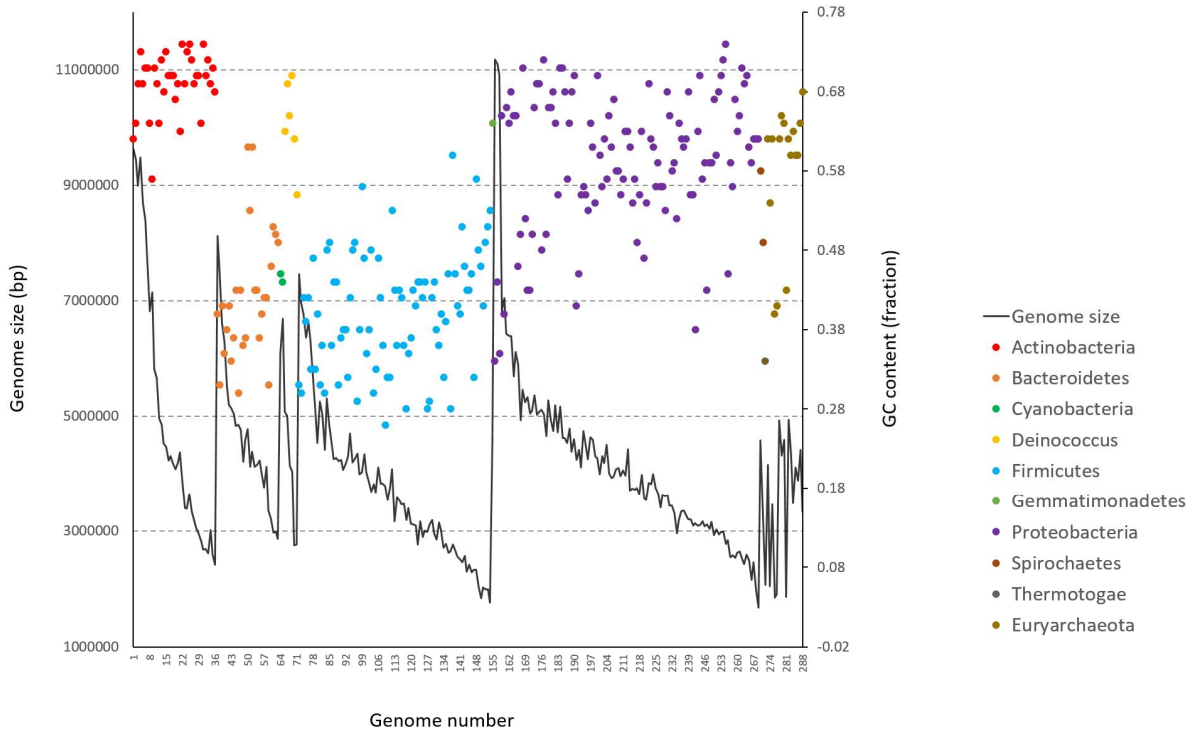


**Figure 1B**- GC content and genome size distributions of the 288 alkaliphile genomes listed in the JGI database. GC content is illustrated with the scatter plot, and units are expression as fractions. Points in the plot are colour-coded to differentiate the different phyla. Genome size distribution is represented by the line plot, and units are expressed in nucleotide base-pairs (bp).

## The Genomic Features of Alkaliphilic Microorganisms

The increasing number of genomes for alkaliphiles allows for comparative genomic studies that reveal the unique genetic features of these organisms. Historically, bacteria from the genus *Bacillus* have been the target of substantial research on the adaptation to alkaliphily, with the genomes of representative strains *B. halodurans* C-125 and *B. pseudofirmus* OF4 being widely studied and characterized (Takami, 2011). Both genomes share a large percentage of genes (1510), as well as 80% conserved synteny and comparable origins of replication (Janto et al. 2011). In turn, the genomes of *B. halodur*ans C-125 and *B. subtilis* were found to share a high number of gene clusters involved in the house-keeping functions such as motility and chemotaxis, sporulation, protein

secretion, main metabolic pathways, and DNA replication (Takami et al. 2000). One big differential factor between these genomes is the number and type of transposable elements. *B. halodurans* contains 112 transposable elements divided into 27 distinct groups compared to the 10 transposable elements in *B. subtilis*, and all of these share significant sequence similarity to transposases and recombinases from species such as *Rhodobacter capsulatus* and *Lactococcus lactis*. In addition, *B. halodurans* C-125 contains 10 unique extracytoplasmic function $\sigma$ factors that might play a role in adaptation to alkaline environments (Takami et al. 2000). A distinct feature of *B. pseudofirmus* OF4 is the presence of two resident plasmids that contain gene clusters for metal acquisition and metal resistance, including P-type metal ATPases, copper chaperones, and cadmium resistance transporters (Janto et al. 2011). Genomic differences between *B. pseudofirmu*s and *B. halodurans* further hint at the more alkaliphilic nature of the former. For instance, *B. pseudofirmu*s contains 13 cation/solute antiporters compared to five in *B. halodurans*, which might contribute to increased capacity to maintain pH homeostasis in the cytoplasm (Janto et al. 2011). This enrichment in proton/cation transporters can also be seen in genomes from other alkaliphiles (Figure 2). Another alkaliphilic bacillus, *Oceanobacillus iheyensis* HTE831, first isolated from deep-sea sediments on the Iheya Ridge (Takami 2002), is a strict aerobe that grows optimally at pH 9.5. This organism contains a 3.6 Mb circular genome with 35.7% GC content, similar tRNA arrangement to *B. subtilis* and no prophages. This genome also contains 3496 CDSs. *O. iheyensis* and *B. halodurans* share 243 putative proteins. Five of these genes encode branched-chain amino acid transporters, which are thought to be important in alkaliphiles due to the conversion into negatively charged glutamic acid, which in turn leads to the acidification of the cytoplasm (Schadewaldt et al. 1995). This idea is further re-enforced by a recent transcriptomics study that shows the up-regulation of branched-chain amino acid transporters in *Halomonas* sp. Y2 under alkaline stress (Cheng et al. 2016).

The obligately haloalkaliphile *Natrialba magadii* is an archaeon that requires a high salt concentration (3.5 M NaCl) and pH (9.5) for optimal growth. The genome consists of four replicons, the largest of which is 3.7Mb in size and has a high GC content (61.42%). Of the 4212 genes coded by

the combined genome, 2387 shared orthologues with the halophilic archaeon *Haloterrigena turkmenica*, which has been isolated from a sulfate saline soil in Turkmenistan (Siddaramappa et al. 2012). The genome of *Natrialba magadii* bears specific features that may be linked to its adaptation to its natural environment. These include the presence of genes coding for proteins involved in the intake and synthesis of osmoprotectant compounds such as trehalose and spermidine, a large cluster of 11 genes coding for putative gas vesicle proteins that would allow for better access to surface oxygen, and genes encoding for metal transport proteins, which would may be involved in metal homeostasis in hypersaline environments (Siddaramappa et al. 2012, Saunders et al. 2010).

The genus *Arthrospira* comprises a group of non-$N_2$-fixing cyanobacteria that grow in carbonate/bicarbonate-rich alkaline lakes and are economically important as food sources. The draft genome of one of the two alkaliphilic cyanobacterial genomes listed in the JGI database, *Arthrospira platensis* C1, was published in 2012 (Cheevadhanarak et al. 2012). This genome shares between 94.93 to 97.43% sequence identity with the other five sequenced *Arthrospira sp.*, which together share a highly conserved core genome. As with many other genomes from alkaliphilic prokaryotes, *Arthrospira platensis* C1 contains several genes encoding $Na^+/H^+$ antiporters, particularly one NapA-type $Na^+/H^+$ antiporter homolog that has been associated with salt and pH homeostasis in alkaline conditions (Cheevadhanarak et al. 2012). Analysis of the genome of another *Arthrospira* sp., *Arthrospira platensis* NIS-39, showed that it contained a large number of genes with adenylate and guanylate cyclase domains that are involved in cAMP and c-di-GMP signal transduction and response to external stimuli. In addition, this genome also contained seven genes for putative $Na^+/H^+$ antiporters, as well as seven $\sigma$ factors from groups 2 and 3, which are involved in responses to environmental stress (Fujisawa et al. 2010).

Despite the limited information on the genomics of eukaryotic alkaliphiles, several biodiversity studies have depicted a large diversity of eukaryotes in alkaline environments, including plankton diatoms, green algae, cryptophytes and haptophytes (Amaral-Zettler 2012; Lanzen et al. 2013;

Keresztes et al. 2012; Grum-Grzhimaylo et al. 2016). Two recently sequenced genomes from the closely related alkaliphilic fungi Acremonium *alcalophilum* ATCC 90507 and *Sodiomyces alkalinus* F11 provide a glimpse into the genetic basis of alkaliphily in eukaryotes (Grum-Grzhimaylo et al. 2018, Perreira et al. 2013). The genomes are 54.42 Mbps and 43.45 Mbps in size, respectively, and encode a comparable number of genes for hydrogen/solute symporters (25 in *S. alkalinus* vs 33 in *A alcalophilum*) sodium/solute symporters (5 in both cases), and solute/hydrogen antiporters (10 in *S. alkalinus* vs 15 in *A. alcalophilum*). Comparison of these genomes in the JGI MycoCosm fungal genomic resource (https://genome.jgi.doe.gov/programs/fungi/index.jsf) shows that more neutrophilic fungi such as *Aspergillus oryzae* and *Plectosphaerella cucumerina* also contain comparable numbers of sodium and hydrogen transporters, and therefore these genes cannot be used as hallmarks for alkaliphily in fungi (Table 1). Instead, both *A. alcalophilum* and *S. alkalinus* show an enrichment of halotolerance proteins with FAD domains, which suggests a strong correlation between halotolerance and alkaliphily in the environments from where these fungi were isolated.

| ***KOG Annotation*** | ***A. oryzae*** | ***P. cucumerina*** | ***S. alkalinus*** | ***A. alcalophilum*** |
|---|---|---|---|---|
| *KOG0672 Halotolerance protein HAL3* | 3 | 2 | 19 | 20 |
| *KOG4126 Alkaline phosphatase* | 3 | 4 | 4 | 2 |
| *OG1397 Ca2+/H+ antiporter VCX1 and related proteins* | 8 | 4 | 4 | 4 |
| *KOG2493 Na+/Pi symporter* | 4 | 6 | 4 | 4 |
| *KOG4505 Na+/H+ antiporter* | 5 | 5 | 2 | 3 |
| *KOG1341 Na+/K+ transporter* | 5 | 5 | 2 | 2 |
| *KOG1650 Predicted K+/H+-antiporter* | 3 | 2 | 2 | 1 |
| *KOG2399 K+-dependent Na+:Ca2+ antiporter* | 1 | 1 | 1 | 1 |
| *KOG0205 Plasma membrane H+-transporting ATPase* | 3 | 1 | 1 | 1 |
| *KOG3126 Porin/voltage-dependent anion-selective channel protein* | 1 | 1 | 1 | 1 |
| *KOG3182 Predicted cation transporter* | 1 | 1 | 1 | 1 |

**Table 1** - Number of genes for proteins associated with alkaliphily in two neutrophile and two alkaliphile fungi according to the eukaryotic orthologous groups (KOGs) annotations obtained from the MycoCosm fungal genomic resource (https://genome.jgi.doe.gov/programs/fungi/index.jsf).

In addition to their genomic heterogeneity, several alkaliphiles have been shown to harbour extra-chromosomal plasmids containing resistance genes and other genetic elements that provide an adaptive edge in the extreme environments where these organisms inhabit (Table 1). For instance, B. pseudofirmus OF4 contains two plasmids, pBpOF4-01 (0.28 Mb) and pBpOF4-02 (0.10 Mb), that code for genes involved in resistance to heavy metals such as cadmium, copper, and mercury (Janto et al. 2011). Similarly, pCHRO.01 (0.37 Mb) from the alkali-tolerant *Chroococcidiopsis thermalis* PCC7203 codes for several metal efflux transporters and multicopper oxidases in addition to toxin-antitoxin systems and DNA translocation proteins. In the case of *Sinorhizobium medicae* WSM419, two of its three plasmids, pSMED01 (1.57 Mb) and pSMED02 (1.24 Mb), represent a large proportion of the genome and code for a broad range of functions in addition to stress adaptation, including proteins involved in energy conversion and amino acid synthesis (Reeve et al. 2010). Alternatively, the two plasmids from the alkalithermophile *Natranaerobius thermophilus* JW/NM-WN-LF primarily code for DNA restriction and modification enzymes as well as a growth inhibition regulator, and are thus not directly involved in stress adaptation (Zhao et al. 2011). Rather, the abundance of transposons, integrases, and other mobile elements in the majority of the plasmids listed in table 1 suggests they also play an important role in both adaptive and non-adaptive plasticity of the genomes of alkaliphiles.

| ***Species*** | ***Plasmids*** | ***Size*** | ***GC %*** | ***Nr. genes*** |
|---|---|---|---|---|
| *Listeria monocytogenes 08-5923* | pLM5578 | 77054 | 36.59 | 79 |
| *Thermobacillus composti KWC4* | pTHECO01 | 149182 | 47.3 | 149 |
| *Kineococcus radiotolerans SRS30216* | pKRAD01 | 182572 | 69.4 | 183 |
| | pKRAD02 | 12917 | 72.3 | 17 |
| *Halobacillus halophilus DSM 2266* | pL16 | 16047 | 43 | 19 |
| | pL3 | 3329 | 36.5 | 2 |
| *Natranaerobius thermophilus JW/NM-WN-LF* | pNTHE01 | 17207 | 34.2 | 17 |
| | pNTHE02 | 8689 | 35.7 | 9 |

| | | | | |
|---|---|---|---|---|
| *Chroococcidiopsis thermalis PCC 7203* | pCHRO.01 | 370830 | 45.1 | 335 |
| | pCHRO.02 | 2779 | 44.1 | 2 |
| *Bacillus pseudofirmus OF4* | pBpOF4-01 | 285222 | 36 | 260 |
| | pBpOF4-02 | 105029 | 35.5 | 113 |
| *Sinorhizobium medicae WSM419* | pSMED01 | 1570951 | 61.5 | 1441 |
| | pSMED02 | 1245408 | 59.9 | 1094 |
| | pSMED03 | 219313 | 60.1 | 149 |
| *Natronococcus occultus DSM 3396* | plasmid 1 | 12939 | 54.8 | 20 |
| | plasmid 2 | 287963 | 61.3 | 238 |

**Table 2 –** Extra-chromosomal plasmids present in representative strains of alkaliphilic bacteria and archaea.

## Genomic insights into the metabolism of Alkaliphiles

Alkaliphilic microorganism are found in a wide range of bacterial, archaeal and eukaryotic phyla, and constitute a functionally diverse group of organisms that play vastly different but inter-dependent ecological roles in their native habitats. For instance, communities in soda lakes are thought to be organised in a complex multi-layered structure, in which the by-products and dead-matter from the primary photoautotrophic cyanobacterial producers at the surface are initially used by a second layer of heterotrophic bacteria from the Firmicutes and Proteobacteria phyla. The metabolic products from these bacteria are subsequently used as substrates for anaerobic organisms that degrade organic matter, producing gases that support the growth of a fourth layer of lithotrophic organisms such as homoacetogens , hydrogenotropic sulfidogens and anoxygenic anaerobic phototrophs (Zavarzin et al. 1999, Sorokin et al. 2014).

As primary producers, cyanobacteria play a crucial role in pioneering and maintaining communities in many extreme environments. Sequence analysis of the alkaliphilic *Arthrospira platensis* showed that it contains a full set of genes for photosystems I and II, as well as variant genes such as cytochrome c550-like and cytochrome $c_6$ (Fujisawa et al. 2010). As a photoautotroph, *Arthrospira*

*platensis* also contains the genes for the common cyanobacterial metabolic pathways, including the pentose phosphate (PP) pathway for primary metabolism of bicarbonate ions, as well as the Calvin cycle for $CO_2$ fixation, which depends on the activity of two enzymes, phosphoribulokinase and RuBisCO. These two proteins are also specific to the PP pathway in light energy conditions, while under dark conditions the glucose-6-phosphate and 6-phosphogluconate dehydrogenases are used (Cogne et al. 2003). Cyanobacteria surviving in alkaline conditions where $CO_2$-concentrations are limited rely on $CO_2$-concentrating mechanisms, which involved $CO_2$ and $HCO_3^-$ uptake by NAD(P)H dehydrogenases and plasma membrane transporters, as well as $HCO_3^-$ conversion into $CO_2$ catalysed in RubisCO containing carboxysome sub-cellular compartments (Price et al. 2008) . A comparative genomics study of all known alkaline cyanobacterial genomes revealed that they all contained the same RubisCO variant, RubisCO B1 (Klanchui et al, 2017). In addition, all the strains analysed contained genes that rncode $CO_2$-concentrating mechanisms, including genes for the $CO_2$-uptake NAD(P)H dehydrogenase type 1 complexes NDH-$1_3$ and NDH-$1_4$, as well as the *ccmKLMNO* cluster, which codes for the structural proteins of the carboxysomes. Conversely, *Arthrospira platensis* NIES-39 and *Arthrospira platensis* sp. *paraca* were shown to contain a gene encoding the high-affinity $HCO_3^-$ transporter StbA, in addition with the low-affinity transporter BicA, which was shared by all the genomes analysed. The presence of both transporters in these strains might provide a selective advantage in highly alkaline environments where $HCO_3^-$ concentrations are high (Klanchui et al, 2017).

Alkaliphilic anaerobes thriving on the products from the primary producers can be expected to metabolize a range of complex carbohydrates. This is the case with the obligately alkaliphilic anaerobe, *Clostridrium alkalicellum*, which was isolated from a cellulose-decomposing community in the Verkhnee Beloe soda lake in Russia. This bacterium was found to be very specialized, with a strictly fermentative metabolism capable of degrading xylan, cellulose and cellobiose into hydrogen, ethanol, acetate and lactate (Zhilina et a. 2005). Other anaerobic heterotrophic bacteria found in soda lakes, such as *Halonatromum saccharophilum*, *Amphibacillus fermentum* and *Amphibacillus*

*tropicus*, isolated from Lake Magadi in Kenya, use a purely fermentative type of metabolism in with mono-, di-, and polysaccharides are catabolized through the fructose biphosphate pathway into acetate, ethanol and $CO_2$ (Garnova & Krasil'nikova, 2003). Microarray studies on another hemicellulose degrading facultative anaerobe isolated from the Wudunur Soda Lake in China, *Bacillus sp,* N16-5, revealed a complex hierarchical pattern of sugar metabolism in which glucose was the preferred substrate, followed by components of complex hemicellulose polysaccharides such as xylan, pectin and galactomannan. Glucose was found to be the primary repressor of expression of gene clusters involved in the degradation of polysaccharides, which are only partially degraded by extracellular glycoside hydrolases before being transported intracellularly by oligopeptide transporters to be further processed (Song et al. 2013). This is thought to be a genetic adaptation to allow responses to the carbohydrate source fluctuations in the environment (Song et al. 2013).

Unlike acetogens from more neutrophilic habitats, acetogenic bacteria found in soda lakes cannot grow chemolithotropically on hydrogen, carbon monoxide and carbon dioxide mixtures, and are trophicaly limited to products from primary anaerobes in the community such as lactate, histidine, and ethanol (Zavarzin et al. 1999). In these bacteria, the Wood-Ljungdahl acetyl-CoA pathway, in which the $CO_2$ generated from the catabolism of the primary substrates is converted to acetate, is coupled with proton-dependent ATP synthesis by generating the electron and proton gradient required by $F_1F_0$-type ATP synthases (Detkova EN & Pusheva MA, 2006). This is the case with the bacteria *Natroniella acetigena* and *Natronincola histidonovorans*, while in *T. magdiensis* the fermentation of arginine through the ornithine cycle and subsequent conversion of carbamoyl phosphate by carbamate kinase is coupled to ATP synthesis (Detkova EN & Pusheva MA, 2006). Genome analysis of the strictly anaerobic and sulfate-reducing bacterium *Thermodesulfovibrio* sp. N1 showed that it lacked the complete TCA and Wood-Ljundahl pathways, and would therefore not be capable of fully oxidizing organic substrates (Frank et al. 2016). Instead, it is able to reversibly decarboxylate pyruvate to acetyl-CoA, which is further converted into acetate by the acetyl-CoA synthetase, generating ATP in the process. Several other dehydrogenases present in the periplasmic

space, such as four [NiFe]-family dehydrogenases that allow for growth with $H_2$ as an energy source, and a formate dehydrogenase that uses formate as a proton donor, contribute to the PMF used to drive proton-coupled ATP synthesis (Frank et al. 2016).

As noted above, alkaline ecosystems also harbour metabolically versatile chemoautotrophic micro-organisms that can feed on a variety of organic and inorganic substrates. *Alkalilimnicola ehrlichii* MLHE-1, a facultative Gram-negative chemoautotroph isolated from the alkaline and hypersaline Mono Lake in the USA, is capable of growing both aerobically and anaerobically with inorganic electron donors such as arsenite, hydrogen and nitrate as the electron acceptor, while also growing heterotrophically on organic acids (Oremland et al. 2002, Hoeft et al. 2007). Genome analysis of this bacterium revealed that, in addition to the RuBisCO genes *cbb*L and *cbb*S, which allow for $CO_2$ fixation through the Calvin cycle, it also contained the CO dehydrogenase operon *coxFEDLSM*, which allows for CO metabolism (Hoeft et al. 2007). Three bacterial strains (A1, B1, and H1) isolated from a serpentinizing site in the USA and phylogenetically related to the genera *Hydrogenophaga* and *Malikia* were also genetically characterized (Susuki et al. 2014). Metabolic profiling of these strains revealed that they have genes required for chemoautotrophic growth on $H_2$, specifically group 2b and 3d [NiFe]-hydrogenases, as well as genes encoding for carboxysome shell proteins, which suggest the ability to employ $CO_2$-concentrating mechanisms for carbon fixation. In addition, strain A1 also contained genes encoding for benzene and phenylalanine/phenyl-acetate degradation, which would facilitate growth in environments with aromatic compounds.

The recent sequencing of the genome of the alkaliphile fungi *S. alkalinus*, originally extracted from soda lakes, also contributed to our understanding of the role that eukaryotic alkaliphiles play in these complex trophic systems. Metabolic profiling of the genome revealed the capacity to grow on xylan from maize, pectins, and monosaccharides (Grum-Grzhimaylo et al. 2018). In addition, *S. alkalinus* was found to express a narrow range of peptidases with strong protease activity at alkaline

pH, which support the hypothesis that it used protein-rich microscopic crustaceans and prokaryotes as primary food sources (Grum-Grzhimaylo et al. 2018).

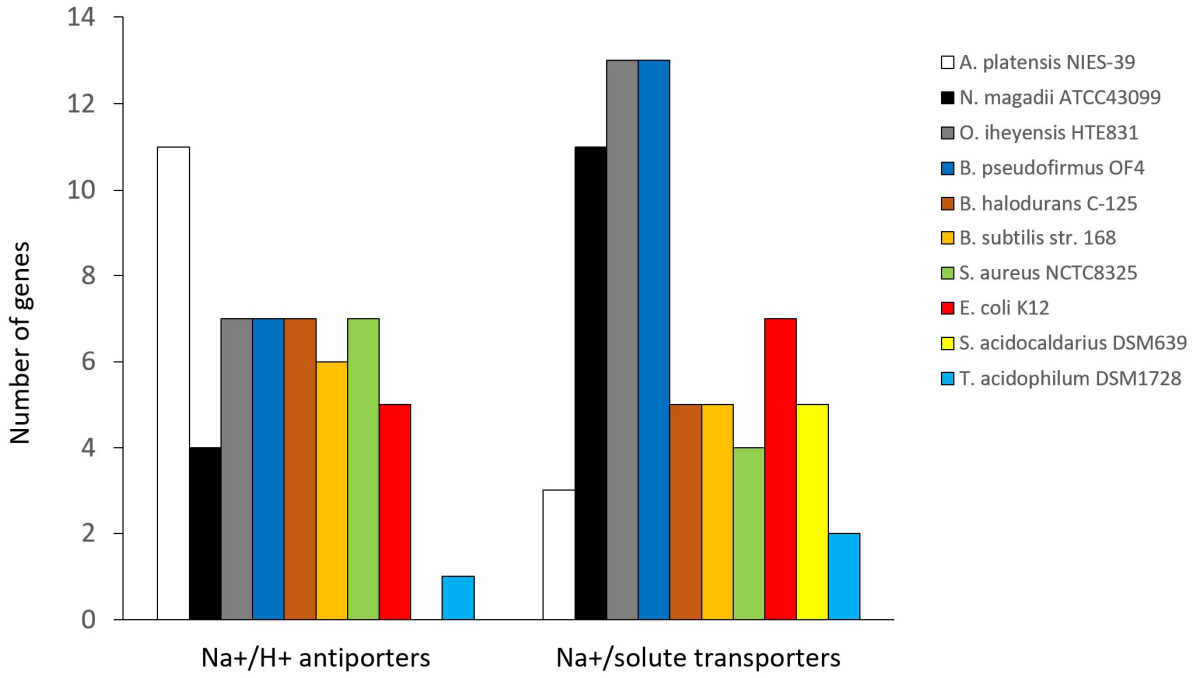


**Figure 2**- Number of genes for $Na^+/H^+$ and $Na^+$/solute symporters and antiporters distributed across the genomes of alkaliphiles, neutrophiles and acidophiles. The alkaliphile genomes are generally found to have a higher number of $Na^+$/solute transporters, while the number of $Na^+/H^+$ antiporters are comparable to their neutrophilic and acidophilic counterparts, with *A. platensis* being an exception. This reverse enrichment in *A. platensis* might be accounted for by the localization of the respiratory apparatus within cytoplasmic vesicles, which might also require the active establishment of a PMF through $Na^+/H^+$ exchange. The genomes were obtained from the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome), and annotated using the EggNOG v4.5.1 mapper (Huerta-Cepas et al. 2017). The alkaliphile species used in this analysis are: *A. plantensis* NIES-39, *N. magadii* ATCC43099, *O. iheyensis* HTE831, *B. pseudofirmus OF*4, and *B. halodurans* C-125. The neutrophile

species used are: *B. subtilis* str. 168, *S aureus* NCTC8325, and *E. coli* K12. The acidophiles used are: *Sulfolobus acidocaldarius* DSM639, and *Thermoplasma acidophilum* DSM1728.

## The Bioenergetics of Alkaliphiles

The bioenergetics of aerobic alkaliphiles revolves around the control of the PMF and SMF across the cytoplasmic membrane. pH homeostasis is regulated by coupling the outwards pumping of protons through the respiratory chain to generate a PMF, with the export of sodium by Na+/H+ antiporters using the PMF. This interplay maintains the conditions required for ion gradient-coupled bioenergetics processes while achieving the acidification of the cytoplasm (Krulwich et al., 2007)

The sodium cycle, in which a sodium motive force (SMF) is established across the membrane to drive bioenergetics processes, has been historically associated with adaptation to alkaline environments where establishing a proton motive force is extremely energy demanding (Skulachev, 1985). However, the existence of alkaliphiles that can grow in alkaline conditions using protons as the coupling ion has since discredited the idea of sodium cycling being an obligate requirement for alkaline adaptation (Hicks & Krulwich, 1995; Mulkidjanian et al., 2009). Nonetheless, the sodium cycle plays an important role in the maintenance of pH homeostasis in a restricted number of alkaliphiles. One of the main antiporters involved in this cycle is the Mrp-antiporter, which was first identified in *B. halodurans* (Ventosa et al. 1998). In *Bacillus pseudofirmus* OF4 the operon for the *mrp* Na+/H+ antiporter contains 7 genes, all of which were found to be essential for the $NA^+$ exclusion and antiport activity (Ito et al. 2001). Two other major components of this cycle are the sodium/solute symporter and the voltage-gate channel associated with a sodium dependent flagellar motor (Krulwich et al. 2001), which is found exclusive in alkaliphiles (Ito et al., 2004). Studies on non-alkaliphilic mutant strains of *B. halodurans* C-125 have revealed the importance of Na+/H+

antiporters, as the alkaliphilic nature of the strain was restored after cloning in a 3.7 kb stretch of DNA containing an Na+/H+ antiporter gene (Horikoshi 1999, Takami et al. 2000). In the Gram-negative bacterium *Alkalimonas amylolytica*, isolated from Lake Chahannor in China, the monovalent sodium/proton antiporter NhaD provided sodium (lithium)/proton antiport activity at pH values above 9 and over a broad range of sodium concentrations, but activity was severely reduced under more neutrophilic conditions (Liu et al. 2005). It is also notable that the genome of the alkaliphile *O. iheyensis* contains 18 genes coding for C4-dicarbozylate carriers, seven of which are also shared by *B. halodurans* (Jamausch et al. 2002), that are commonly associated with Na+ and H+ symport activity.

A recent transcriptomics study of the alkaliphile *Halomonas* sp. Y2 revealed that it differentially expresses distinct Na+/H+ antiporters in response to different stresses. For instance, expression of the Mrp transporter did not vary across different pHs, while its absence negatively impacted the organism's resistance to Na+, Li+, and K+ ions. By comparison, the Ha-NhaD2 transporter, which is homologous to the NhaD antiporter from *Alkalimonas amylolytica*, was up-regulated with increased pH, suggesting that it plays a more significant role in pH homeostasis than Mrp, under alkaline stress (Cheng et al. 2016). In addition to the canonical sodium/proton antiport, some members of this superfamily also show functional versatility. This is the case of the antiporter Ap-NapA1-2 from the halotolerant alkaliphilic cyanobacterium *Aphanothece halophytica*, which shows the capacity to replace proton uptake by potassium uptake (Wutipraditkul et al. 2005).

Due to the reversed proton gradient in alkaliphiles, where the cytoplasmic pH is more acidic than the alkaline environment, processes that require a PMF, such as motility, are severely compromised (Krulwich, 1995). To circumvent this limitation, alkaliphiles use SMF-driven motors to achieve motility (Hirota et al. 1981). In *B. halodurans* C-125 and *B. pseudofirmus* OF4, MotPS functions as the sole sodium-dependent stator for the flagellar motor (Takami et al. 2000), while *O. iheyensis* also contains the proton-coupled MotAB stator (Takami et al. 2002). The difference between these

species might be due to the more halotolerant nature of the latter. Conversely, *Bacillus claussi* KSM-K16 contains a set a genes with high homology to MotAB from *B. subtilis* that can couple both sodium and protons for motility (Terahara et al., 2008). Another sodium channel, NaChBac, was found to play a major role in the pH homeostasis of *B. halodurans* C-125 and *B. pseudofirmus* OF4, particularly in conditions where sodium and solute concentrations are low (Ren et al., 2001, Fujinami et al. 2009).

## The alkaliphilic respiration chain

As noted above, the bioenergetics of alkaliphiles depends on the delicate balance between pH homeostasis and the PMF. The acidification of the cytoplasm has the detrimental effect of significantly reducing the PMF across the membrane. For instance, the PMF of the facultative alkaliphile *Bacillus* sp. TA2.A1 shifts from -164 mV to -78 mV when pH is changed from 7.5 to 10 (Olsson et al. 2003). Despite this apparent limitation, aerobic alkaliphiles are adapted to cope with a reduced proton electrochemical gradient (Hicks & Krulwich. 1995). Examples of high pH-specific adaptations include the over-expression of cytochromes in some strains when grown in high pH environments (Guffanti et al. 1986), and the fact that the redox midpoint potential of these cytochromes is markedly lower than for their neutrophilic counterparts, as is the case with the cytochrome-b from *Bacillus firmus* RAB (Kitada et al. 1983). The respiratory chain of *B. pseudofirmus* OF4 is similar to that in other *Bacillus* species, containing two NADH dehydrogenases, a succinate dehydrogenase, as well as cytochrome *bd* and cytochrome $caa_3$. Other than the elevated cytochrome expression, the other components of the respiratory chain maintain constant expression levels across different pHs (Hicks & Krulwich. 1995).

The archaeon *Natrialba magadii* contains a full arsenal of proteins that allow for efficient respiration and oxidative phosphorylation. These include the operon atpHIKECFAB, encoding a proton-coupled ATP synthase, genes encoding for type II and mitochondrial NADH dehydrogenases,

as well as genes for the cytochrome c-type terminal oxidase subunits I and II and for the cytochrome ubiquitol oxidase I on II. Conversely, the *N. magadii* genome does not encode acytochrome bc1, which is predicted to couple reduced quinone to the electron carrier halocyanin in halophilic archaea. The presence of several two-component signal transduction systems as well as 3 rhodopsin homologue genes and two loci encoding for chemoreceptors suggests that *N. magadii* is capable of sensing and responding to a variety of light and chemical signals (Siddaramappa et al. 2012).

## The link between respiration and ATP synthases

Despite the low bulk PMF generated in high pH environments, alkaliphilic *Bacillus* species exclusively use proton-coupled $F_1F_0$-ATP synthases, contrary to some anaerobic and fermentative bacteria which use Na+-coupled ATP synthases. Evidence showing that respiration supports maximal ATP synthesis at high pH, while artificially imposed diffusion gradients fail to energize the ATP synthase, suggests that the cytochrome complexes involved in the generation of bulk electrochemical gradient during respiration play a crucial role in the oxidative phosphorylation in alkaliphiles (Krulwich et al. 2007). Models suggest that physical interactions between the terminal oxidases of the respiration chain, such as cytochrome-$caa_3$, and the ATP synthase would allow for protons to be retained in the membrane instead of dissipating into the extracellular environment (Krulwich et al. 1998). The operon coding for the ATP synthase in *B. pseudofirmus* OF4, the atp operon, contains an extra gene upstream of the conventional genes coding for the ATP synthase complex and the putative chaperone atpI. This gene, atpZ, has several homologues in other Firmicutes and, together with atpI, is hypothesized to form a divalent cation channel for the import of $Mg^{2+}$. Magnesium is required for the formation of the transition state between ADP and ATP in the $F_1$ catalytic moiety of the ATP synthase, and might contribute to charge compensation during pH cytoplasmic homeostasis in alkaliphiles (Ko et al. 1999; Hicks et al. 2003). In addition, structural studies have shown that proton-coupled synthases from alkaliphiles contain specific amino acid compositions that allow ATP

synthesis at high pH values. For example, a lysine residue at position 180 of the alpha subunit of the ATP synthase from *Bacillus* sp. TA2.A1 was shown to facilitate proton capture at high pH (McMillan et al. 2007). Multiple alignment studies between the alpha subunits of alkaliphiles and neutrophiles identified additional motifs that are distinct in alkaliphiles, in particular two conserverd methionines (M171 and M184) in the membrane portion close to the proton pathway that affect growth on malate when replaced by the neutrophilic counterparts (Preiss et al. 2015). The ATP synthase of *B. pseudofirmus* OF4 contains a consensus motif AxAxAxA in the N-terminal helix of the c-subunit that is conserved across alkaliphilic Bacilli, and is suggested to play an important role in the correct assembly of the c-subunit rotor, as indicated by the inability of mutants with a GxGxGxG substitution to produce ATP (Liu et al. 2009). Another example of how the respiration chain is linked with ATP production in alkaliphiles comes from the cyanobacterium *Arthrospira platensis*, in which the $F_0F_1$ ATP synthase is co-localized with the photosynthetic complex in thylakoid vesicles in the cytoplasm (Pogoryelov et al. 2005; Liberton et al. 2006). Since the cytoplasmic pH of this cyanobacterium is actively maintained at neutral pH through the activity of Na+/H+ antiporters, the PMF across the thylakoid membranes is much more akin to that found in neutral environments and thus favourable for oxidative phosphorylation (Hicks et al. 2010; Pogoryelov et al. 2003).

Due to the “reverse” transmembrane pH characteristic of alkaline habitats, it was expected that alkaliphiles would express sodium-coupled ATP synthases to take advantage of the greater SMF generated during pH homeostasis. However, only alkaliphilic anaerobes such as *Clostridium paradoxum* have been found to contain a Na+-coupled ATP synthase. However, this enzyme exhibits low ATP synthesis activity and is thought to rather play a role in the establishment of an SMF for sodium-coupled solute uptake and motility (Ferguson et al. 2006). A reason for the preferential use of proton-coupled ATP synthases in most alkaliphiles might relate to the overwhelming pressure to maintain pH homeostasis, as the proton uptake during ATP synthesis contributes to the acidification of the cytoplasmic environment (Hicks et al. 2010). This suggestion is also re-enforced by the fact that most alkaliphile ATP synthases exhibit very low or absent hydrolytic activity. For instance, the

ATP synthase from the thermoalkaliphilic *Bacillus* sp. TA2.A1 contains two salt-bridges in the beta subunit that prevent the protein from rotating in the ATP hydrolytic direction, thus preventing the outwards flux of protons to the bulk medium (Stocker et al. 2007).

***NADH and succinate dehydrogenases***

Studies of *B. pseudofirmus* have demonstrated that it contains two distinct types of NADH dehydrogenases, NDH-2A, and NDH-2B (Hicks & Krulwich, 1995). NDH-2A shows significant orthology with the type-2 NDH from *Bacillus subtilis*, YjlD (37% identity), while NDH-2B shows significant homology (56%) to a putative NADH dehydrogenase from *Halobacillus dabanensis* that has been shown to couple NA+/H+ antiport activity to the respiration chain (Liu et al. 2009). Both dehydrogenases are localized in the cytoplasm and show very different spectrums of activity, with NDH-2A being predominantly active against NADH, while NDH-2B also shows activity against NADPH and d-NADH with ferricyanide being the primary acceptor. It is hypothesized that NDH-2A plays the primary role in providing the entry point for electrons to the respiratory chains (Liu et al. 2009).

*Bacillus* YN-1 was found to contain a type 2 NDH homodimer which contains one FAD and shows homology to a thioredoxin reductase from *Escherichia coli* (Xu et al. 1991). In turn, the NDH-2 from the thermoalkaliphilic bacterium *Caldalkalibacillus thermarum* is an FAD and NADH utilizing homodimer composed of subunits with both membrane-anchoring and catalytic domains, where binding sites for NADH and quinone do not overlap (Heikal et al. 2014).

**Cytochromes**

In addition to the antiporters, *B. halodurans* also contains two bo3-type cytochrome c oxidase genes that are absent from the neutrophilic *B. subtilis* (Takami, 2011). C-type cytochromes from alkaliphiles can be distinguished by being low midpoint potential electron carriers (+50 to +100 mV) compared to their neutrophilic counterparts (+180 mV to +250 mV), and by having low isoelectric points (Hicks & Krulwich, 1995). *B speudofirm*us was shown to express four distinct heme-containing

membrane peptides, all of which have counterparts in *B. subtilis* (Hicks & Krulwich, 1995). One of these is subunit II of the terminal oxidase cytochrome $caa_3$, which in *B. firmus* RAB and *B. pseudofirmus* OF4 is composed of 3 subunits (I-III). In *B. pseudofirmus* OF4, the cytochrome-$caa_3$ complex coded by the *cta* operon, with *cta*C-F coding for the four subunits (Quirk et al. 1993), while *cta*A and *cta*B are involved in heme A synthesis (Brown et al. 2002; Throne-Holst & Hederstedt, 2000). In *B. pseudofirmus* OF4, c-type cytochromes are found to be upregulated at high pHs (Hicks & Krulwich, 1995). Another terminal oxidase found in *B. pseudofirmus* is cytochrome-bd, which is only expressed during stationary phase at high pHs and therefore probably does not play a role in alkaliphily (Hicks & Krulwich, 1995). By comparison, *Bacillus* YN-2000 expresses cytochrome-aco as its terminal oxidase, in which hemeC and the protoheme O are directly involved in the transfer of electrons from cytochrome-c553 to oxygen, while heme A might operate as an electron reservoir (Yumoto et al. 1991).

Another defining feature of the surface proteins of alkaliphiles is enrichment of neutral and acidic residues. For example, the exposed loops of the membrane-bound cytochrome-caa3 in *B. pseudoformis* have a lower number of basic residues than its neutrophilic homologues (Hicks & Krulwich, 1995). Analysis of predicted protein isoelectric point (pI) distribution across the genomes of alkaliphiles also shows a skew towards lower pH values, indicating a preference for acidic amino acids (Figure 3).

While Gram-positive alkaliphiles contain a single cytoplasmic membrane in which most cytochromes are embedded, Gram-negative alkaliphiles have a periplasmic space between the outer and inner membranes and are thus able to express soluble cytochromes. The facultative alkaliphile *Pseudomonas alcaliphila* AL 15-21$^T$ expresses three soluble cytochrome c proteins, namely cytochrome-c552, c-554 and c-551, with cytochrome-c552 making up more than 60% of the total soluble cytochrome c content (Matsuno & Yumoto, 2015). The midpoint potential of cytochrome-c552 in this bacterium is similar to that of neutrophilic cytochromes-c (+218 mV at pH 9 and 10),

which suggests that the bioenergetic state of the periplasm of *Pseudomonas alcaliphila* is akin to of its more neutrophilic counterparts. Despite this, cytochrome-c552 was shown to play an important role for growth of this bacteria at high pH by working as a proton reservoir in the periplasmic space to compensate for the low proton concentrations in the extracellular medium (Matsuno & Yumoto, 2015).

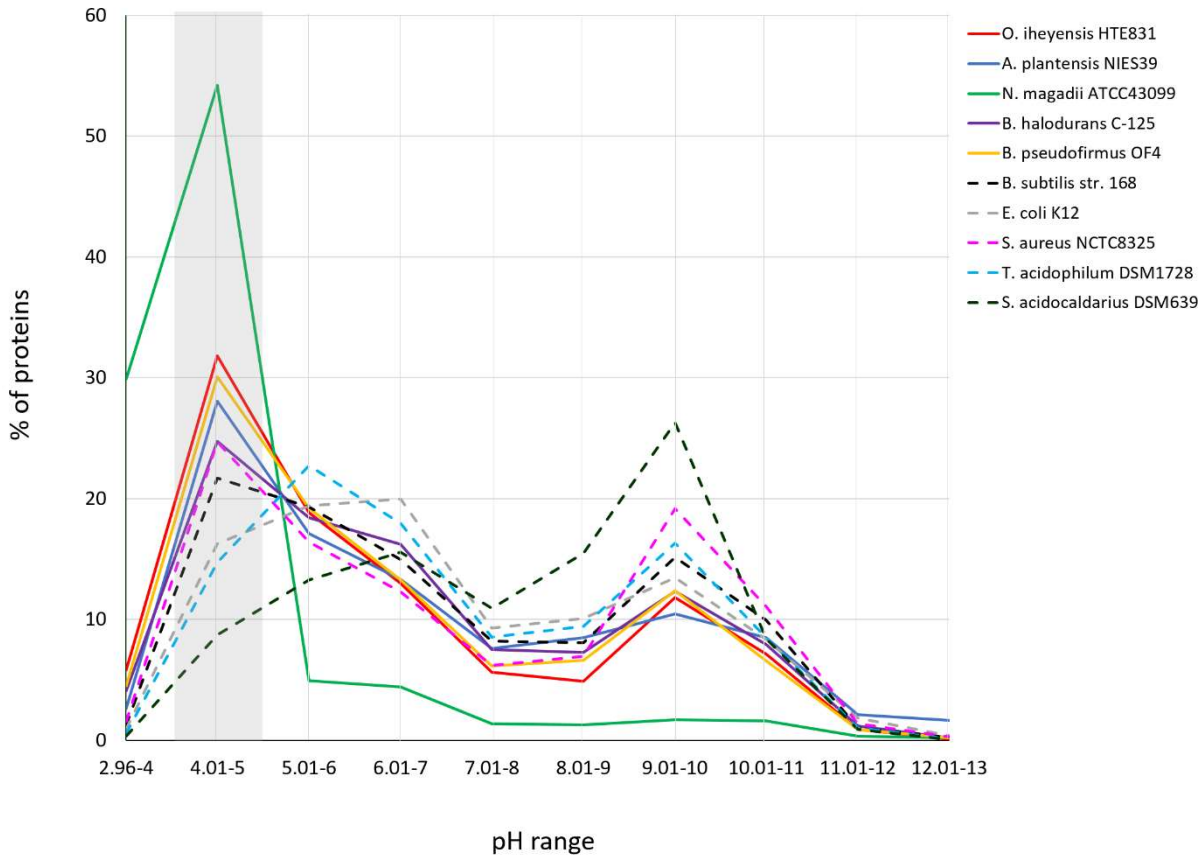


**Figure 3-** Protein *pI* distribution across alkaliphilic, neutrophilic, and acidophilic genomes. Alkaliphile genomes are represented by the solid plot-lines, while neutrophile and acidophile genomes are represented by the dashed plot-lines. The alkaliphilic *pI* skew towards low pH values is illustrated by the gray area across the plot. The pI values for proteins of each genome were calculated using the Sequence Manipulation Suite server (Stothard, 2000).

## Concluding remarks

Alkaliphiles exist in one of the accepted 'extreme environments', where selective pressures preclude most other organisms. In alkaline niches such as soda lakes and saline freshwater, a combination of abiotic pressures, including low $CO_2$ and metal ions concentrations, low proton gradient, and high salinity, impose a thermodynamic burden on essential biological functions such as carbon fixation, motility and oxidative phosphorylation. Despite apparent thermodynamic limitations, alkaliphiles comprise a diverse group of microorganisms with divergent genetic origins, metabolic requirements and functional capabilities. This genetic diversity, observed across eukaryotic, archaeal, and bacterial phyla, suggests that alkalinity is not a 'narrow' driver of the evolution of organisms, in that alkaliphilicity is a derived property across a very wide taxonomic space.

Alkaliphiles share two main features: the ability to maintain pH homeostasis, and the ability to perform bioenergetics processes in an environment with an inverse, or "reversed", chemical gradient. Accordingly, many alkaliphile genomes are enriched in genes for cation/proton antiporters and cation/substrate symporters. The enrichment of such transporters reflects the need to balance both PMF and SMF in order achieve pH homeostasis and fuel energetically expensive processes such as substrate acquisition and chemotaxis. In addition alkaliphile genomes encode a large number of membrane-localized proton and electron-retaining proteins such as cytrochrome oxidases, which allow for the maintenance of a PMF across the membrane that favours efficient proton-coupled ATP synthesis. Some level of metabolic specialization can also be observed as a possible response to secondary pressures exerted in an alkaline environment. For instance, alkaliphilic cyanobacteria express genes involved in $CO_2$-concentration mechanisms, which allow for efficient carbon fixation in environments where the levels of $CO_2$ are low. Other alkaliphilic bacteria resort to using other gases and inorganic compounds such as $H_2$ and sulphites as energy sources, although such trophic characteristics are not limited to high pH environments. In turn, heterotrophic aerobes and aerobes utilize on the products of the primary autotrophs. As is typical of all complex communities, microbial communities in alkaline niches live as multi-layered tiers of microorganisms that are functionally and

metabolically interdependent, where such interdependency is expressed as a high level of genetic diversity.

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