Transcriptomic analysis reveals how a lack of potassium ions increases Sulfolobus acidocaldarius sensitivity to pH changes

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Extremely acidophilic microorganisms (optimum growth pH of ≤ 3) maintain a near neutral cytoplasmic pH via several homeostatic mechanisms, including an inside positive membrane potential created by potassium ions. Transcriptomic responses to pH stress in the thermoacidophilic archaeon, Sulfolobus acidocaldarius were investigated by growing cells without added sodium and/or potassium ions at both optimal and sub-optimal pH. Culturing the cells in the absence of added sodium or potassium ions resulted in a reduced growth rate compared to full-salt conditions as well as 43 and 75 significantly different RNA transcript ratios, respectively. Differentially expressed RNA transcripts during growth in the absence of added sodium ions included genes coding for permeases, a sodium/proline transporter and electron transport proteins. In contrast, culturing without added potassium ions resulted in higher RNA transcripts for similar genes as a lack of sodium ions plus genes related to spermidine that has a general role in response to stress and a decarboxylase that potentially consumes protons. The greatest RNA transcript response occurred when Sulfolobus acidocaldarius cells were grown in the absence of potassium and/or sodium at a sub-optimal pH. These adaptations included those listed above plus osmoregulated glucans and mechanosensitive channels that have previously been shown to respond to osmotic stress. In addition, data analyses revealed two co-expressed IclR family transcriptional regulator genes with a previously unknown role in the Sulfolobus acidocaldarius pH stress response. Our study provides additional evidence towards the importance of potassium in acidophile growth at acidic pH.

INTRODUCTION

Extreme acidophiles represent a group of microorganisms with an optimum pH for growth of ≤ 3. Low pH environments can be of natural origin such as solfatara, as well as anthropogenic that include areas associated with mining (Dopson, 2012). Sulfolobus acidocaldarius is mainly found in terrestrial solfatara springs and is an aerobic thermoacidophilic crenarchaeon that grows on organic substrates in pH ~1.8 and temperatures ranging between 70–80°C. Sulfolobus acidocaldarius DSM639 is used as a model organism for the Crenarchaeota phylum. In addition to its available genome sequence (Chen et al., 2005), considerable progress has been made in establishing molecular biology tools such as knock-out techniques and shuttle vectors that allow genetic studies (Berkner & Lipps, 2008; Wagner et al., 2012).

One of the distinguishing properties of acidophiles is their structural/functional features that establish an electrochemical gradient across their cytoplasmic membrane that is utilized to generate ATP (Dopson et al., 2002). Acidophiles utilize a number of methods to maintain a near neutral intracellular
pH. These include an internal positive membrane potential suggested to be generated by cations (Cox et al., 1979); primary proton transporters during electron transport (Ferguson & Ingledeiw, 2008); secondary proton transporters (Fütterer et al., 2004); proton-tight membranes [based on tetra-ether lipids in archaea (van de Vossenberg et al., 1998)]; and proton-consuming reactions (Mangold et al., 2013). High external concentrations of anions such as chloride are particularly toxic to acidophiles as the cells import competing ions (including protons) to balance the osmotic pressure that dissipates the trans-membrane proton gradient causing cell death (Suzuki et al., 1999). Mechanisms for combating osmotic stress in acidophilic microorganisms have recently been reviewed (Watkin & Zammit, 2015) and include increasing cell turgor pressure by the production of compatible solutes, uptake of potassium via a channel sensitive to osmotic tension as well as the production of membrane proteins. These mechanisms likely result in the exclusion of sodium ions from the cell. The inclusion of potassium ion transporters in the acidophile response to both osmotic and pH stresses appears to highlight its importance.

The reversed, as compared to neutrophiles, inside positive membrane potential is proposed to constitute an important adaptive mechanism for life in highly acidic conditions by creating an electrochemical barrier to proton influx [reviewed in Slonczewski et al. (2009)] and to counter hyperosmotic stress (Cao et al., 2011; Follmann et al., 2009; Reed et al., 1985). Several reports provide circumstantial evidence that the acidophilic membrane potential is created by potassium, and to a lesser extent, sodium ions. These include that cations (potassium ions are the most effective) are required for respiration-linked proton extrusion in Sulfolobus species (Schäfer, 1996); generation of a membrane potential in Acidithiobacillus thiooxidans requires the presence of cations, with potassium being the most efficient (Suzuki et al., 1999); and the presence of numerous putative cation transporters found in the genome of acidophiles (Chen et al., 2005; Fütterer et al., 2004; She et al., 2001; Tyson et al., 2004). However, the mechanism for membrane potential generation is poorly understood (Slonczewski et al., 2009).

Potassium is the most abundant cation in the cytoplasm of all cells, reflecting its deep-rooted compatibility with cellular structures throughout evolution (Dibrova et al., 2015; Kuo et al., 2005). The role of potassium ions is suggested to have been retained as it would be too ‘energetically demanding’ to alter (Mulkidjanian et al., 2012). Potassium influx is regulated by potassium-uptake mechanisms or via simple passive diffusion (Kuo et al., 2005). In contrast, the accumulation of intracellular sodium causes toxic effects due to its higher surface charge density with respect to potassium ions. This results in cytoplasmic [K⁺]>[Na⁺] in almost all actively metabolizing organisms (Mulkidjanian et al., 2012).

RNA-seq methods utilizing next-generation sequencing enable detailed transcriptome profiling (Wang et al., 2009). However, the large amount of data often defies straightforward intuitive interpretation and requires novel bioinformatics and statistical methods to process (Janes & Yaffe, 2006; Wang et al., 2009). Examples of such methods include principal component analysis (PCA) and hierarchical clustering (HC) to identify potentially co-expressed genes over the different combinatorial growth conditions. In addition, Bayesian network (BN) analysis can be used to infer regulatory linkages between mRNA abundances (Friedman, 2004; Hurley et al., 2012; Sachs et al., 2005; Yu et al., 2004).

Here, to address the role of protons, potassium ions and sodium ions in acidophiles, we followed a well-established RNA-seq data processing and analysis pipeline to study the S. acidocaldarius transcriptomics response, while methodically limiting the available potassium and sodium ions and applying pH-stress.

METHODS

Strain and growth conditions. Triplicate cultures of S. acidocaldarius DSM639 were inoculated in 250 ml Erlenmeyer flasks at an optical density at 600 nm (OD₆₀₀) of 0.005 and grown at 70 °C in a shaking incubator (150 rpm) with different media inducing pH-stress. All solutions were prepared with MilliQ ultrapure water and HPLC-grade chemicals. Modified mineral salts media (MSM) (Dopson et al., 2004) and 0.1% (wt/vol) starch were adjusted to the desired pH with H₂SO₄ and auto-claved before addition of 0.22 µm sterile filtered trace elements (TE) (Dopson & Lindström, 1999). In order to apply the desired stress induced by potassium and sodium depletion, chemicals in the MSM and TE were replaced to exclude Na or K. Accordingly, the standard components of MSM and TE were replaced with equivalent amounts of substitutes such that final concentrations of chemicals other than Na or K were maintained as constant. The substituted chemicals in the MSM (as required) were: KCl with HCl and K₂HPO₄ with H₃PO₄ for potassium-depleted media, and Na₂SO₄ with H₂SO₄ for sodium-depleted media. For sodium depletion, the chemicals in the trace elements were replaced as follows: Na₂MoO₄-2H₂O with H₂MoO₄, and Na₃SeO₃ with K₂SeO₃. Final potassium and sodium concentrations were measured on a Sherwood Model 410 Flame Photometer with samples diluted to <10 ppm of K⁺ and Na⁺. The calculated standard concentrations of potassium and sodium in the MSM were 20 and 1.90 mM, respectively, while actual measured values are presented in Supplemental File 1 (available in the online Supplementary Material).

RNA extraction and sequencing. With the exception of one full salt culture [(+)-Na⁺/(+-)K⁺ (pH 2.0)] that was taken in the stationary phase, all cells were collected in late exponential growth phase (OD₆₀₀ from 0.62 to 0.90). The cells were harvested by mixing 100 ml of culture with an equal volume of 4 °C-cold salt-free MSM, centifuging at 10 000 g for 10 min and washing twice with 4 °C-cold salt-free MSM. The resulting pellet was immediately stored in RNA stabilizing solution (RNaIater, Ambion) at ~20 °C.

Duplicates (chosen from the three cultures based upon those that reached the highest optical density or randomly if all cultures grew to the same level) from each growing condition were processed for RNA extraction and sequencing. RNA stabilizing solution (RNaIater, Ambion) was removed by careful pipetting from ~20 °C-stored cell pellets and replaced with 1 ml lysis buffer (RNeasy Midi kit (Qiagen)) and glass beads. Cells were then lysed with three cycles of freeze-thaw in liquid nitrogen alternated with bead beating. Samples were shaken using a vortex-adaptor until the sample was completely melted. Total RNA was purified using RNeasy Midi kit (Qiagen) according to the
manufacturer’s instructions except that the S. acidocaldarius lyase was centrifuged at 5000 g until all of the liquid flowed through the column filter. To eliminate contaminant DNA, DNase treatment was applied using Turbo DNA-free Kit (Ambion). Nanodrop and Qubit analyses were used to assess the quality and quantity of extracted RNA, together with products from PCR and RT-PCR on a 2 % agarose gel with control primers for archaeal 16S RNA gene [oligonucleotides arc344f (5'-ACGGGGGCGCAAGCGCGG-A-3') and arc915r (5'-GTTGCGT-CCCCGGGCAATTCC-3')] in order to confirm the absence of DNA and presence of amplifiable RNA (data not shown). Total RNA samples (70 µL of concentrations of 70–250 ng µL⁻¹ in RNAse-free water with a RNA integrity number (RIN) value ≥ 9.0 and an optical density 260 nm/280 nm ratio > 2.0) were processed by SciLifeLab for RNAseq without ribosomal depletion on one Illumina HiSeq 2500 lane in high-output mode running 2 x 100 bp pair end libraries.

**RNA-seq data processing.** The Galaxy web-based platform was used to process each individual RNA-seq dataset separately using the default settings (Goecks et al., 2010). In particular, the quality of the raw reads was assessed using FASTQ Groomer version 1.0.4 (Blankenberg et al., 2010) and reads with any base quality under 20 were removed. Raw reads were then mapped onto the S. acidocaldarius DSM 639 reference genome as downloaded from Ensembl genome database (CP000077.1, Sulfolobus acidocaldarius_dsm_639. GCA_000012285.1.29.dna.chromosome) using Bowtie 2 version 2.0.2 aligner (Langmead et al., 2009) in default, paired-end mode. BAM-to-SAM version 1.0.3 (Li et al., 2009) was used to transform BAM files into SAM and HTSeq-count version 0.6.1 (Anders et al., 2014) was used in union mode to count reads mapping to the gene features as defined in the gene annotation file available at Ensembl (Sulfolobus acidocaldarius_dsm_639.GCA_000012285.1.29.dna.chromosome.Chromosome.fa). The final data matrix contained counts for 2274 genes across six different sample conditions.

Statistical methods for data analyses were utilized to identify different mRNA abundances or isoforms between conditions, while controlling for false discovery rates by taking biological variation into account (Anders & Huber, 2010; Anders et al., 2012; Robinson et al., 2010). Data analyses were performed in R version 3.1 using the Bioconductor edgeR version 3.6.2 package for empirical analysis of digital gene expression data (Robinson et al., 2010). Raw count reads were removed by retaining those with at least 1 read per million in at least three samples and normalized using the TMM method, i.e. weighted trimmed mean of M-values to the reference (Robinson & Oshlack, 2010). Different mRNA abundances between all the pairwise group comparisons were identified via computing gene-wise exact tests for false discovery rates by taking biological variation into account for false discovery rates by taking biological variation into account. The analysis was performed by applying the procedures prcomp, cmdscale and hclust in R. The HC results were represented in a tree-like structure (dendrogram) composed of branches that group genes according to their expression similarities over the different conditions: the more similar the expression of two genes, the closer their position in the dendrogram. Two measures were used to account for the relatedness and specificity of links inferred by HC. The height h of the tree branch that contains a set of genes reflects the degree of similarity of their co-expressed patterns (relatedness). The smaller the height of the branch, the more uniform the group. The size of a cluster that contains the gene group of interest was used as a measure for specificity: if a gene group clustered in a given branch, the number of other genes clustered in the same branch indicated how specifically those genes were co-expressed with other genes considered in the analysis. This number is given in absolute values (n, the number of genes in the same cluster of the gene of interest) and in percentage (n %) with respect to the whole set of genes that compose the entire dendrogram.

In addition, BN analysis [BNfinder-2.0.4 (Dojer et al., 2013)] was applied to identify small gene regulatory networks from read counts. BN analysis requires the definition of a variable subset of ‘regulators’. In this work, two different subsets were defined as follows. The first subset included genes that were differentially expressed more than once among all pairwise condition changes. The second subset included transcriptional regulators among all significantly different mRNA abundances. BN analysis was carried out in parallel with the two regulator subsets and the corresponding output was used to retrieve potential links between genes. BNfinder-2.0.4 was run with default parameters except for the threshold weight for the observations (d), which was increased in order to increase prior and posterior probabilities, consequently making the procedure more sensitive to the assignment of activation or inhibition links between genes (the smaller the d-value the more reliable the assigned link). Finally, we also report the score assigned to a pair of interacting genes according to the String database (Franceschini et al., 2013). This database was used to search for gene interactions predicted by the methods mentioned above.

**RESULTS AND DISCUSSION**

**Sulfolobus acidocaldarius batch growth cultures**

Initial experiments with various concentrations of Na⁺ and K⁺ (without pH stress) did not significantly affect the S. acidocaldarius growth rate (data not shown). It was therefore decided to culture the cells in the complete absence of added Na⁺ and K⁺ in the medium (Fig. 1). Despite the best efforts to remove Na⁺ and/or K⁺ by using ultra-pure water and HPLC-grade chemicals, it was not possible to completely remove these cations from the media and the final concentrations were reduced to 4 µM Na⁺ and ≤16 µM K⁺ (Supplemental File 1).

All of the S. acidocaldarius cultures grew at a similar rate except those under acid stress (pH 1.6) and the replicate flasks in the presence of Na⁺ but no added K⁺. The Hill slopes extrapolated from a four-parameter logistic equation showed that the growth rates in the presence of full salt at pH 2.0 were reduced by 29 % when cultured at pH 1.6 and by 56 % in the absence of Na⁺ and K⁺ ions at pH 1.6 (Fig. 1). The lack of a strong growth rate effect when potassium and/or sodium was removed (in the absence of additional pH stress) suggested the S. acidocaldarius cells had a very good scavenging ability to accumulate K⁺ as well as efficient systems to remove Na⁺ from the cell.

**Global transcriptomic changes**

All of the cultures yielded RNA libraries of sufficient quality for sequencing except one of the duplicate cultures at pH 1.6 and in the absence of both Na⁺ and K⁺ ions (Supplemental File 2). The average number of raw reads across the
The data above and below the diagonal represent the number of genes that were up- and down-regulated under the different conditions, respectively. The genes were considered up- and down-regulated when their transcripts were $\log_2$ (treated/reference) > 1 or < −1.

Table 1. Up- and down-regulated genes under different growth conditions

<table>
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<tr>
<th>Condition 1</th>
<th>Condition 2</th>
<th>$\text{(+)}$Na$^+$/$\text{(+)}$K$^+$</th>
<th>$\text{(+)}$Na$^+$/$\text{(-)}$K$^+$</th>
<th>$\text{(-)}$Na$^+$/$\text{(+)}$K$^+$</th>
<th>$\text{(-)}$Na$^+$/$\text{(-)}$K$^+$</th>
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certain that *S. acidocaldarius* has a type I, proton-translocating NADH dehydrogenase [or if it only has a non-proton-translocating Type II complex (Schäfer, 2004)]. In addition, *S. acidocaldarius* has a novel cytochrome bc₁ analogous complex (Hiller et al., 2003) as well as three terminal oxidases: DoxBCE, SoxABCDL and the SoxM complex (Reimann et al., 2013). SoxABCDL oxidase and the SoxM complex are linked to oxygen availability (Simon et al., 2002), a condition that was not tested in this study. RNA transcripts for uroporphyrin III methyltransferase (Saci_2200) were higher in the presence of full salt compared to the absence of sodium. The product of this gene has an unknown role in osmotic stress in halophiles (Krisko et al., 2014), and its function in *S. acidocaldarius* was not experimentally confirmed. The ratio of CDP-diglyceride synthetase RNA transcripts involved in membrane biosynthesis was higher under full salt, possibly as its activity is stimulated by potassium ions (Langley & Kennedy, 1978). Relative RNA transcripts for permeases (genes Saci_1059 & 2151) with an unknown function were increased in the absence of potassium ions compared to full salt [(+Na⁺/−K⁺) vs (+)Na⁺/(−)K⁺] (Figs 3 and 4a and Supplemental Files 4 and 5). An increase in RNA transcripts was also observed for subunit D of the proton-extruding archaeal succinate dehydrogenase (Saci_0979). Removing potassium compared to full salt also resulted in a large increase in RNA transcripts for the polyanime, spermidine synthase (Saci_0643). Spermidine stabilizes ribosomes for protein synthesis at high temperature (Howland, 1995; Londei et al., 1986) and also has a role in other stress responses. Finally, a higher ratio of isocitrate dehydrogenase (Saci_2375) RNA transcripts involved in the oxidative stress response was observed when potassium ions were not added to the media. However, how a reduced potassium concentration may cause oxidative stress remains unknown.

The RNA transcript response to removing only either sodium or potassium ions compared to removing both cations [(−)Na⁺/(+K⁺) vs (−)Na⁺/(−)K⁺ and (+)Na⁺/(−)K⁺] was similarly minor (Fig. 3 and Supplemental Files 4 and 5). An increased ratio of RNA transcripts encoding peroxiredoxin (Saci_2227) involved in the acidophilic, archaean oxidative stress response (Dopson et al., 2005) was observed in the absence of both cations compared to just no added sodium. In addition, a lack of both sodium and potassium ions compared to just a lack of potassium, resulted in higher RNA transcripts counts for a sodium/proline symporter (Saci_0383) possibly needed to maintain a higher intracellular [K⁺]>[Na⁺] (Mulkidjanian et al., 2012).

**Fig. 2.** Global transcriptomics samples profiles. (a) MDS plot of samples based on their genome-wide gene expression showing global response to different sample conditions, and (b) heat-map of Pearson correlations among different samples. Both (a) and (b) reveal the largest transcriptomics response from full salt to no salt plus pH stress.
Fig. 3. Summary of different mRNA abundances in the presence of low sodium and/or potassium (a) and pH stress (b) suggested as having a role in pH homeostasis. Only genes that code for proteins suggested as having a role in pH homeostasis and discussed in the text are listed, while the full results are given in Supplementary File 4. Superscript numbers (in parentheses) link the up- and down-regulated genes (red and blue, respectively) with the different growth conditions. The first column in the boxes corresponds to *S. acidocaldarius* gene IDs, the second column to log2-FC in the corresponding growing conditions, and the third column to the arCOGs gene function.
Fig. 4. pH homeostatic responses to reduced concentrations of sodium and potassium ions and pH stress induced by growth at pH 1.6. (a) pH homeostatic responses to reduced sodium and potassium ions in comparison to full salt. Increased RNA transcript number as a result of either low sodium or potassium are in red, increased ratios of RNA transcripts in full salt are illustrated in blue, while processes that were not influenced are in black; (b) pH homeostatic responses to no added sodium or potassium ions in comparison to full salt and pH stress induced by growth at pH 1.6. Changes as a result of either low sodium or potassium are in red, increased ratios of RNA transcripts in full salt plus pH stress are illustrated in blue, while processes that were not influenced are in black. Proton translocation by the complex I is marked with a question mark as it is uncertain whether type I NADH dehydrogenases are present; and (c) pH homeostatic responses to full salt compared to no salt and pH stress induced by growth at pH 1.6. Increased ratios of RNA transcripts as a result of full salt and no salt plus pH stress.
**RNA transcripts as a response to potassium or sodium ion removal**

A comparison of growth with no added sodium versus medium without added potassium [(−)Na⁺/(+)K⁺ vs (+)Na⁺/(−)K⁺] resulted in 14 and 48 genes with statistically higher RNA transcript ratios, respectively (Fig. 3 and Supplemental Files 4 and 5). The RNA transcripts with higher ratios in the absence of sodium included a sodium/proline symporter (Saci_0383) and two permeases (Saci_2127 and 2028). The greater response to a lack of potassium included the cytochrome b homologue of the SoxABC complex (Saci_2087); three ribosomal subunits suggesting a greater need for protein biosynthesis possibly to combat a lack of potassium (Saci_0758, 0962, and 0620); and uroporphyrin III methyltransferase (Saci_2200) previously shown to be involved in the response to osmotic shock in halophiles (Krisko et al., 2014).

**RNA transcripts as a response to reduced potassium and sodium ions**

A comparison of RNA transcript ratios between full salt versus no salt [(+)Na⁺/(+)K⁺ vs (−)Na⁺/(−)K⁺] resulted in a gene response similar to the result for pH stress described above with 42 statistically different RNA transcript ratios (Fig. 3 and Supplemental Files 4 and 5). RNA transcripts with statistically valid higher fold changes in the presence of full salt included two copies of the SoxABC complex cytochrome b homologue (Saci_2087 and 2260), a permease (Saci_2111) and uroporphyrin III methyltransferase (Saci_2200). In contrast, growth under reduced concentrations of both potassium and sodium ions resulted in increased RNA transcript ratios for the sodium/proline symporter (Saci_0383); two permeases (Saci_2151 and 1521); 4-aminobutyrate aminotransferase (Saci_2137) potentially involved in glutamate decarboxylase consumption of protons; and N-acetylglucosamine-1-phosphate uridyltransferase (Saci_1199) involved in cell membrane biosynthesis. Removing both potassium and sodium ions was insufficient to cause a major stress response. This suggested that an adequate concentration of K⁺ ions was imported from the cytosol to generate an inside positive potential, that these ions were not responsible for the internal positive membrane potential in acidophilic archaea, or that additional pH homeostatic mechanisms (e.g. membranes resistant to influx of H⁺ (van de Vossenberg et al., 1998)] were sufficient to maintain a near neutral pH (Slonczewski et al., 2009).

**RNA transcripts as a response to removal of either potassium and/or sodium ions compared to growth under pH stress**

Removing sodium ions versus full salt at a sub-optimal growth pH of 1.6 [(−)Na⁺/(+)K⁺ vs (+)Na⁺/(+)K⁺ pH 1.6] had a similar effect to removing potassium ions [(+)Na⁺/(−)K⁺ vs (+)Na⁺/(+)K⁺ pH 1.6] with 41 and 37 RNA transcripts with statistically valid fold changes, respectively (Figs 3 and 4b and Supplemental Files 4 and 5). Removing sodium ions resulted in an increased RNA transcript ratio for genes encoding a permease (Saci_2129) not seen in the other conditions. The lower effect of removing sodium ions may have been due to the need to maintain the cytoplasmic Na⁺ ion concentration below that of K⁺ ions (Mulkidjian et al., 2012) or that K⁺ ions maintain pH homeostasis via the internal positive membrane potential (Slonczewski et al., 2009).

Increased RNA transcript ratios were detected during pH stress to potentially sustain increased proton export via the NADH dehydrogenase (Saci_2346); two permeases (Saci_2110 and 1755); and membrane changes via an acetyl-CoA acetyltransferase (Saci_1121) and N-acetylglucosamine-1-phosphate uridylyltransferase (Saci_0196). Removing potassium ions versus full salt at a growth pH of 1.6 resulted in increased RNA transcript ratios for a permease (Saci_1036); spermidine synthase (Saci_0643); and 4-aminobutyrate aminotransferase (Saci_2137) potentially involved in proton consuming decarboxylase reactions.

**RNA transcripts as a response to growth without sodium or potassium ions under pH stress**

The comparison between full salt versus no salt plus pH stress [(+)Na⁺/(+)K⁺ vs (−)Na⁺/(−)K⁺ pH 1.6] is based on a single biological sample for the no salt plus pH stress. As a result, the evaluation utilizes descriptive statistics based on log₂-fold changes and not P-values, and we have added log₂-fold change values to the genes discussed below. This comparison resulted in 245 genes with statistically different numbers of RNA transcripts, many of which have previously been reported to be related to cellular homeostasis (Figs 3 and 4c and Supplemental Files 4 and 5). RNA transcripts with higher fold changes in the presence of full salt included 13 permeases and 25 membrane-related proteins suggesting alterations in relation to the different growth conditions. In addition, two subunits of the NADH dehydrogenase [Saci_1702 (log₂-FC = −5.8) and Saci_2344 (log₂-FC = −5.9)], the SoxABC complex cytochrome b homologue [Saci_2087 (log₂-FC = −1.9)] and the flavoprotein subunit of the succinate dehydrogenase [complex II; Saci_0982 (log₂-FC = −6.8)] not identified in the other conditions had higher relative RNA transcripts (negative log₂-FC ratios meaning higher expression in the presence of full salt). This may be due to pH stress inhibiting all electron transport, as has been observed in the acidophile At. caldus (Dopson et al., 2002). A higher ratio of RNA transcripts in the presence of full salt was also observed for a Kf-type K⁺/H⁺ antiporter [Saci_0364 (log₂-FC = −1.6)] that leads to acidification of the cytoplasm. Transcript ratios for the Kf-type K⁺/H⁺ antiporter increased from 1.2 and 1.3 for no salt plus pH stress versus no sodium or potassium, respectively and up to 1.6 for no salt plus pH stress versus no salt. This increase in transcript ratio highlights the importance of the potassium antiporter in pH homeostasis. A second Kf-type
K⁺/H⁺ antiporter (Saci_2186) also had a higher relative RNA transcript ratio (6.2-fold) in no potassium versus no salt plus pH stress \((+\text{Na}^+)/(-\text{K}^+)+\text{pH} 1.6\). This system was likely identified due to very low expression of the gene in the absence of salt with pH stress, and again emphasizes the importance of potassium. Four glycosyltransferase genes \([\text{Saci}_0275 (\log_{2}-\text{FC} = -5.83), 1011 (\log_{2}-\text{FC} = -1.19), 1805 (\log_{2}-\text{FC} = -7.06)\) and 1909 \((\log_{2}-\text{FC} = -1.11)\] had higher RNA transcript ratios in the presence of salt. These proteins synthesize osmoregulated periplasmic glucans in bacteria that respond to osmotic stress (Bohin, 2000) while Saci_1909 has been implicated in the production of extracellular polysaccharide in \(S.\ acidocaldarius\) biofilms (Orell et al., 2013). The potential role of these glucans during growth with full salt is unknown as they have not been identified in archaea, and previous studies have shown that the genes are downregulated with increased external osmolarity, and \(S.\ acidocaldarius\) does not have a periplasm. A small-conductance mechanosensitive channel (Saci_1914) had a \(\log_{2}-\text{FC}\) increase of 7.8 in the presence of full salt. Small-conductance mechanosensitive channels open and close in response to stretching of the membrane, protecting the cell against osmotic shock (Martina et al., 2014), and their role in archaea is well established (Kloeda & Martinac, 2002).

RNA transcripts with increased ratios in the absence of sodium or potassium ions plus stress generated by growth at pH 1.6 included five permeases, four proteins associated with the cell membrane, and archaeal succinate dehydrogenase subunit D (Saci_0979). Both a sodium/proline symporter (Saci_0383) and an ABC-type sodium efflux protein (Saci_0945) had higher RNA transcript ratios. These proteins would appear to be acting in conflict, but a possible explanation is be that the sodium/proline symporter is required for sodium uptake and that the efflux protein maintains the cytoplasm \([K^+]>[Na^+]\). A second small-conductance mechanosensitive channel (Saci_0230) had a slightly higher RNA transcript level \((\log_{2}-\text{FC} \approx 1.10)\) when compared to full salt. The dissimilar responses of the two channels (compared to Saci_1805 described earlier) suggested they open and close under different conditions to maintain pH balance. Two glycosyltransferase genes (Saci_1249 and 1916) that synthesize osmoregulated periplasmic glucans also had higher RNA transcript ratios in the absence of salt plus pH stress. Increased glycosylation of the \(S.\ acidocaldarius\) cytochrome \(b_{558/566}\) is attributed to increased complex stability at low pH (Eichler & Adams, 2005; Hettmann et al., 1998; Zähringer et al., 2000), while high glycosylation of S-layer components protects the cell against osmotic and pH stress (Peyfoon et al., 2010). The glycosyltransferase genes with increased RNA transcripts may enable a similar function in this study.

**Network analysis**

The network construction is data-driven (i.e. inferred based on our data with the software Bnfinder-2.0.4) to identify the most probable links between genes that best explains the observed RNA levels (Supplemental File 6). A purely objective Bayesian network analysis is too demanding computationally and therefore, a set of genes (termed ‘regulators’) was used to restrict the computation. These regulators included both genes involved in multiple conditions of the applied stress (i.e. genes that appeared to play a role in our experiments based on RNA data) and known transcriptional regulators, since they orchestrate the genetic adaptations at the DNA level in different growth conditions.

Network analyses suggested interactions between several families of transcriptional regulators and the genes suggested by the RNA transcriptomics to be involved in \(S.\ acidocaldarius\) growth in the absence of sodium and potassium ions (Fig. 5). These included two IclR-family transcriptional regulators (Saci_2136 and Saci_0752); the Saci_1787 ArsR family transcriptional regulator; the CopG/Arc/MetJ family transcriptional regulator; an AbfB family transcriptional regulator (Saci_1955); and finally, a predicted HTH transcriptional regulator (Saci_2296). IclR-family transcriptional regulators control gene expression in processes including the glyoxylate shunt and aromatic degradation [reviewed in Molina-Henares et al. (2006)]; ArsR regulators regulate arsenic resistance genes but also have other functions such as regulation of metal-requiring processes [reviewed in Osman & Cavet (2010)], and CopG/Arc/MetJ family transcriptional regulators have been implicated in, for example, anoxic redox control [Arc; (Alvarez & Georgellis, 2010)]. However, the inferred role of these regulators in pH stress has not been reported and requires further experimentation to be confirmed.

Several genes encoding electron transport proteins were positively or negatively clustered depending on the conditions, suggesting different roles during culture with low concentrations of sodium and potassium plus pH stress. Cytochrome \(b_{558/566}\) subunit A (Saci_1858) and subunit B (Saci_1859) displayed opposite regulation during pH stress: Saci_1858 had a higher RNA transcript ratio while Saci_1859 had a lower ratio (Fig. 5), indicating the different subunits may have different roles under these conditions. Two genes encoding the NADH dehydrogenase complex I (Saci_2344 and Saci_1702) clustered very closely with cytochrome \(b\) subunit of the SoxABCD complex (Saci_2087) and a succinate dehydrogenase gene (Saci_0982). Another succinate dehydrogenase subunit (Saci_0979), which is potentially controlled by the ArsR regulator (Saci_1787), showed a very different response compared to Saci_0982, suggesting they may have different functions in the absence of cations or under pH stress, but this requires further experimentation to confirm. Finally, as shown in the presence of full salt versus no salt, the SoxABCD complex cytochrome \(b\) homologue subunits (Saci_2087 and 2260) had higher RNA transcript ratios. This suggested different sub-networks of interaction for the SoxABCD complex cytochrome \(b\) homologue (Saci_2260) as a consequence of changing external conditions.
Links associated with membrane transport and alterations identified by the modelling (Fig. 5) include linking of the sodium/proline symporter Saci_0383 to the Saci_1762 ABC transporter not previously suggested as having a role in pH homeostasis. In addition, uroporphyrinogen-III methylase (Saci_2200) had positive links to a divalent metal transporter (Saci_2239), a negative link to the Saci_2186 Kef type K$^+$/H$^+$ transporter, and a connection to the archaeal/vacuolar Na$^+$/H$^+$ ATPase subunit Saci_1552. Further regulatory links for the Saci_1059 permease include a positive link to a further permease (Saci_2151) and a link by HC to phosphoesterase phosphohydrolase (Saci_0321) that has only a known general function and has not been linked to pH stress. These links support the suggested roles of the transport proteins in the response to a lack of potassium or sodium ions and/or pH stress.

The connections for the cytoplasmic proteins pirin (Saci_2297), decarboxylase (Saci_2055) and spermidine synthase (Saci_0643) are shown in Fig. 5. In addition, pyruvate dehydrogenase Saci_0136, which was consistently up-regulated in the absence of salt together with pH stress (except when Na$^+$ was present), was found in the same cluster together with a gene coding for pirin-related protein.

**Fig. 5.** Summary of interactions between cellular components predicted by network analysis in the stress response. (a) Connections between transcriptional regulators and other cellular components. (b) Summary of electron transport genes predicted to be involved in the stress response induced by growth in the absence of potassium and/or sodium ions. (c) Connections between genes involved in membrane transport and membrane adaptations. The signs (+/-) indicate positive/negative interactions between genes as predicted by BN analysis, while connections without a positive or negative sign were predicted by HC.
CONCLUSIONS

The RNA transcript data suggest that \textit{S. acidocaldarius} responds to culturing in the absence of sodium and/or potassium ions plus pH stress by increasing transcripts coding for multiple potential pH stress homeostasis mechanisms, including primary and secondary proton pumps, mechanosensitive channels, permeases, membrane changes and a decarboxylase. In addition, a lack of potassium ions increased the sensitivity of \textit{S. acidocaldarius} to pH changes, supporting its possible role in forming an internal positive membrane potential. The network analysis allowed inference of connections between genes without prior knowledge, such as the role of the two IclR family and ArsR transcriptional regulators that were suggested to play major roles in regulating the \textit{S. acidocaldarius} pH stress response. To confirm the hypotheses of the role of potassium ions in \textit{S. acidocaldarius} pH homeostasis, further experiments utilizing sodium and/or potassium transporter gene knockouts should be carried out.

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