Microbial Cooperative Molecular Strategies Enabling 1,2-Dichloroethane Detoxification in Low pH Aquifers

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Abstract

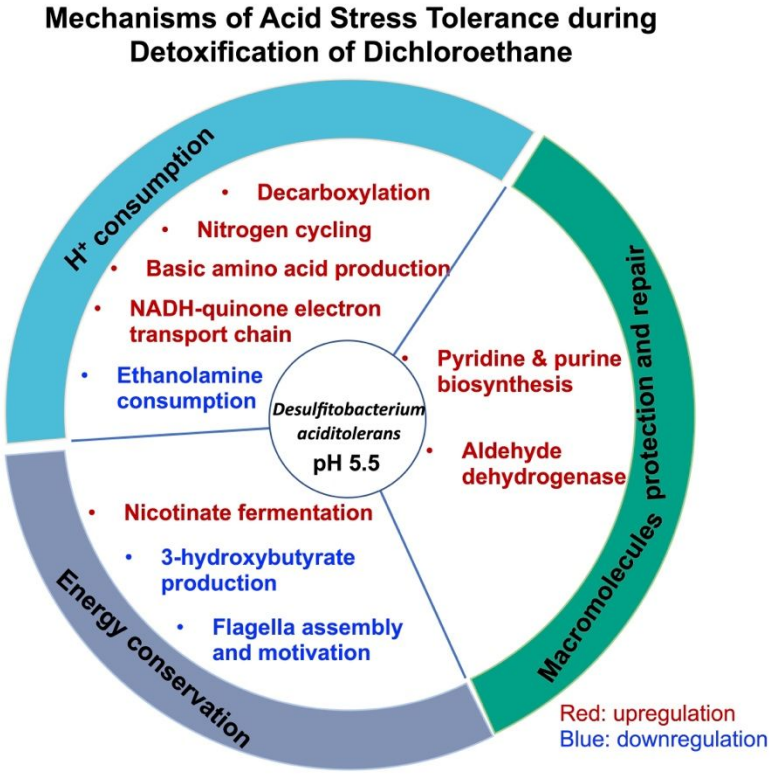
Organohalide-respiring bacteria (OHRB) play a critical role in the bioremediation of halogenated pollutants. However, their activity is often compromised in naturally occurring acidic environments that impose substantial physiological stress, with only a few OHRB remaining functional. The mechanisms enabling these acid-tolerant OHRB to thrive under low pH environments remain largely unexplored. *Desulfitobacterium* sp. strain AusDCA is one such acid-tolerant OHRB, capable of respiring 1,2-dichloroethane to ethene across a broad pH range, with dechlorination activity still observed at pH 5.0, though at a rate 86.9% lower than at pH 7.0. Proteomic analysis revealed a shift in electron transfer strategy under acid stress at pH 5.5, with downregulation of hydrogenases and upregulation of NADH-quinone oxidoreductase, suggesting reliance on NADH as the primary electron donor. Other acid tolerant mechanisms to mitigation of intracellular proton included the upregulation of decarboxylases, nitrogen cycling genes, and basic amino acid production, as well as reduced ethanolamine consumption. Energy conservation adaptation involved upregulation of nicotinate fermentation and downregulation of 3-hydroxybutyrate production and flagellation. These adaptations enabled strain AusDCA to maintain respiratory activities and support reductive dehalogenation under acidic conditions, offering insights into microbial adaptation and potential strategies for low-pH bioremediation.

KEY WORDS:

Acidic tolerance, organohalide respiring bacteria, reductive dehalogenation, *Desulfitobacterium*

SYNOPSIS:

Understanding the specific adaptations that enable survival and dehalogenation function in low pH environments provides critical insights into the metabolic and regulatory networks of organohalide-respiring bacteria, which can be leveraged to enhance the efficiency of bioremediation in acidic sites.



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INTRODUCTION

The extensive historical use of halogenated compounds, such as trichloroethene and dichloroethane (DCA), in various industrial and agricultural applications has precipitated their ubiquitous distribution in the environment[1, 2]. These compounds pose significant environmental and health risks due to their recalcitrance, toxicity, and propensity to contaminate soil and groundwater[1, 3-5]. Organohalide respiration, a microbial process wherein certain bacteria utilize halogenated compounds as terminal electron acceptors, has emerged as an environmentally friendly and cost-effective approach for the detoxification of organohalide contaminants. These bacteria, known as organohalide-respiring bacteria (OHRB) can reductively dehalogenate these compounds, thereby transforming them into less harmful or non-toxic end products [6, 7]. While the majority of research on organohalide respiration has been conducted under neutral pH conditions, contaminated sites often exhibit a spectrum of pH values, including acidic environments.

In natural and contaminated aquifers, pH levels can vary widely, often ranging from \ acidic (pH ~5.0) in organic-rich or anthropogenically impacted zones to neutral or mildly alkaline environments (pH ~7.0–8.0) typical of carbonate-buffered systems. Acidic conditions in aquifers are more prevalent than previously recognized; for instance, nationwide surveys in the United States have documented extensive acidification, with over 81% of wells investigated in the Northern Atlantic Coastal Plain between 1988 and 2009 exhibiting pH values below 6.5 in both surface and groundwater sources [8]. Acidic conditions can profoundly influence the metabolic activities of microorganisms, thereby directly impacting the efficacy of bioremediation strategies for organohalide pollutants [9-12]. Elucidating the phylogenetic niches of acid-tolerant or acidophilic bacteria capable of dehalogenating organohalide pollutants, as well as the

mechanisms by which these microbes adapt to and thrive in acidic environments, can yield insights into their metabolic versatility and resilience, offering novel opportunities for bioremediation.

To date, a phylogenetically diverse array of organohalide respiring bacteria (OHRB), including genera such as *Dehalococcoides*, *Dehalobacter*, *Dehalogenimonas*, *Dehalobium*, *Geobacter*, *Desulfitobacterium*, and *Sulfurospirillum*, as well as uncharacterized genera from marine and engineered systems, have demonstrated the capability to respire a variety of organohalide pollutants [13-24]. However, the majority of these identified OHRB perform dehalogenation predominantly under neutral conditions, with dehalogenation activity generally abating at pH 6.5, or at most, pH 6 [11, 12]. Only a few strains, including *Sulfurospirillum* strains ACSTCE and ACSDCE, have shown the ability to partially dechlorinate tetrachloroethene to trichloroethene or dichloroethenes at pH 5.5, and strain AusDCA was the only OHRB known to dechlorinate 1,2-DCA to environmentally benign ethene via dihaloelimination under even more acidic conditions (pH 5.0) [25]. These three strains represent the only known OHRB capable of performing organohalide dehalogenation under acidic conditions. However, it remains unknown how these OHRB survive and adapt to acidic conditions.

In this study, we aimed to explore the survival mechanisms and effects on organohalide respiration of OHRB in low pH conditions, marking a pioneering examination of organohalide respiration under acidic environment. We evaluated the dechlorination performance of strain AusDCA across different pH levels. Subsequently, we identified the novel phylogenetic identity of strain AusDCA through genomic analysis with its closest phylogenetic relatives. Additionally, we inferred the mechanisms enabling strain AusDCA to thrive in acidic environments via comparative proteomic analysis conducted at pH 5.5 and pH 7.0. These findings from this study

advance our understanding of organohalide respiration in acidic environments and highlight the potential of strain AusDCA for the bioremediation of chlorinated solvents under such conditions. By elucidating the metabolic pathways and adaptive mechanisms of strain AusDCA, we provide a foundation for developing effective bioremediation strategies tailored to acidic contaminated sites. This research underscores the importance of considering pH as a critical factor in bioremediation and opens new avenues for exploring the diversity and capabilities of OHRB in diverse environmental contexts.

MATERIALS AND METHODS

Cultivation and chemical analyses

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany) at the highest purity available unless specified otherwise. Strain AusDCA was grown in 60 ml anaerobic serum bottles containing 30 ml mineral salts medium (DCB1), purged with 20% CO_2 and 80% N_2 as headspace as previously described; cultures were amended with pyruvate (10 mM) as the sole carbon source and electron donor and 1,2-DCA (concentration as indicated in text) as the sole electron acceptor. Cultures at pH 7.0 and 7.5 were buffered using $NaHCO_3$ (30 mM) and at lower pH (4.5-6.5) were buffered using MES (25 mM) as previously described [25]. An important consideration in this study is the use of different buffering agents—$NaHCO_3$ at pH 7.0 and MES at pH 5.5—to maintain the desired pH conditions. Although pH remains the primary variable of interest, we acknowledge that buffer-specific properties such as ionic strength and buffering chemistry could have had a modest influence on cellular responses. This potential source of variation does not undermine the main findings but is noted here to aid in the comprehensive interpretation of the data.

All the kinetics studies at each pH were performed in triplicates with a 5% inoculation. 1,2-DCA and its metabolic product, ethene, were measured using an Agilent gas chromatograph (GC7890) equipped with a flame ionization detector and a DB-5 column (10 m × 0.25 mm ×0.25 μm; J&W Scientific USA) via manual injection of headspace samples as previously described [26]. The temperature program was initiated at 50 ºC for 1 min, increased to 120 ºC at 15 ºC /min and held for 1 min.

Molecular analysis

For kinetics analysis, the cell abundance of strain AusDCA during dechlorination was monitored using quantitative real-time PCR (qPCR) targeting 16S rRNA gene-based *Desulfitobacterium* genus specific primer Dsb1299F/Dsb1448R [27] divided by six, as strain AusDCA possesses six rRNA operons. Genomic DNA was extracted from 1 ml culture using the DNeasy Blood and Tissue Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The qPCR was conducted on an ABI 7500 Fast System using the SYBR green reporter (SensiFAST™ SYBR Lo-ROX Kit, BIOLINE, London, UK) [28, 29]. The thermal cycler program was 95 ºC for 3 min, followed by 40 cycles of 94 ºC for 15s, 55-60 ºC for 30 s, depending on the annealing temperature of primers, and 72 ºC for 10s, and then 30s at 72 ºC. Plasmids carrying target genes were used as standards for qPCR. qPCR standards were constructed using the pGEM-T Easy Vector system (Promega, Madison, WL, USA) according to the manufacturer's instruction and extracted using the QIAprep Spin Miniprep kit (QIAGEN® GmbH, Hilden, Germany).

Genome sequencing, assembly, annotation and comparative analyses

Genomic DNA (gDNA) was extracted from strain AusDCA using the Qiagen Genomic-Tip 100/G (Qiagen, GmbH, Hilden, Germany). The quality and concentration of the extracted

gDNA were assessed by Qubit and the availability of large DNA fragments (>25kbp; required for the generation of long-read PacBio libraries) was determined by electrophoresis. Illumina short-read library preparation and whole genome shotgun sequencing of gDNA was performed on an Illumina NovaSeq PE150 platform, and a PacBio sequel II DNA CLR library was prepared and sequenced on a PacBio Sequel system II by Novogene, Singapore.

De novo assembly of the strain AusDCA genome from the PacBio CLR subreads and Illumina reads was done using both the St. Petersburg genome assembler (SPAdes v3.15.2) [30] and Canu v2.2 [31]. The draft assemblies were polished using Pilon v1.24 and contigs were scaffolded using SSPACE-LongRead [32]; the polished scaffolds from the two assemblers were reconciled with Metassembler v1.5 [33].The assembled genome of strain AusDCA comprised a single circular elements of 4540274 bp. ORFs were identified using Prodigal v2.6.3 by default parameters and translated CDS were annotated against the Swiss-Prot [34]and NCBI RefSeq [35] databases using BLAST v2.13.0+ with a e-value cut-off of 1e-5; rRNAs and tRNAs were identified in the sequence using RNAmmer v1.2 [36] and tRNAscan-SE v2.0.12 [37], respectively.

Average nucleotide identity (ANI) and pairwise percentage of conserved proteins POCP analysis of strain AusDCA and other members from its phylogenetically close genera in the *Peptococcaceae* Family (i.e., *Desulfitobacterium*, *Desulfosporosinus*, and *Acidodesulfobacillus*) [38]. The 16S rRNA gene sequence of strain AusDCA was aligned with 16S rRNA gene sequences of other *Peptococcaceae* Family mined from Genbank using Muscle v5 [39]and a phylogeny was inferred using MEGA X with Maximum-likelihood clustering methods with a bootstrap of 1000. Genome classification was performed using the Genome Taxonomy Database Toolkit (GTDB-Tk, version 2.3.2) with the Genome Taxonomy Database (GTDB release R08-RS214) [40].

Proteomic sequencing and analysis

Proteomic expression profiles under pH 5.5 and pH 7.0 by strain AusDCA were elucidated by proteomics analyses. The progress of DCA degradation was continuously monitored to determine appropriate sampling points for proteomic analysis. Cells of strain AusDCA grown under both pH 5.5 and pH 7.0 conditions were harvested when approximately 60–70% of DCA had been depleted, ensuring sampling at a comparable physiological stage of active degradation. Cells from four biological replicates for each pH condition were harvested by centrifugation (10,000 ×g, 20 min, 16 °C) from 30 ml cultures amended with 1,2-DCA (0.43 mM) and resuspended in ammonium bicarbonate buffer (100 mM). Proteins were extracted from resuspended pellets by three freeze-thaw cycles (-80 °C followed by 40 °C for 1 min), followed by ultrasonication for 30 s. The extracted proteins were digested with trypsin. Peptide samples were desalted using ZipTip-μC18 material (Merck Millipore) and later assayed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoLC system (Dionex Ultimate 3000RSLC; Thermo Fisher Scientific), as previously described [41]. Proteins were identified against the protein database annotated from complete genome of strain AusDCA with Proteome Discover (v2.4, Thermo Fisher Scientific) using the SequestHT search engine. Protein and peptide abundances were calculated by label-free quantification (LFQ) based on intensity values using the Minora node implemented in Proteome Discoverer. LFQ intensities are dimensionless values representing relative protein abundance based on normalized spectral intensities across samples. Normality of protein production data was assessed using the Shapiro-Wilk test applied to each protein across biological replicates within each treatment group. Over 80% of the proteins in both pH conditions passed the

normality test (p-value > 0.05), indicating an approximately normal distribution. This justified the use of parametric two-sample T-tests for differential expression analysis.

Data availability

The assembled and annotated (Novaseq PE150 and PacBio Sequel II CLR) strain AusDCA have been deposited in gene bank under Bioproject SAMN42994840 with accession number CP165781. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [42] partner repository with the dataset identifier PXD059385.

RESULTS

Strain AusDCA performs efficient dechlorination under acid stress

Strain AusDCA has demonstrated robust acidic tolerance across a broad pH range compared to other OHRB. Specifically, the dechlorination of 1,2-DCA (~0.45 mM) to ethene via dihaloelimination was observed within 20 days at pH levels between 7.5 and pH 5.0 (Figure 1a), although the rate of which was significantly influenced by pH. The optimal dechlorination activity was observed at a neutral pH of 7.0. At this pH, the dechlorination rate reached 107.1±12.4 μM DCA dechlorinated/day, which is 3.9 times higher than the rates at slightly alkaline (pH 7.5) and acidic (pH 6.5) conditions, at 27.2±4.2 and 27.3±2.1 μM DCA dechlorinated/day, respectively (Figure 1b). Even as the pH decreased to 5.5 and 5.0, the dechlorination persisted, maintaining approximately half the rates (14.3±2.1 and 14.0±3.1μM DCA dechlorinated/day) of that observed at pH 7.5 and 6.5 (Figure 1b). The sustained capability of strain AusDCA to detoxify DCA to non-toxic ethene at low pH enables it as an ideal candidate bioinoculant to clean up relevant acidic sites.

The correlation between the growth of strain AusDCA and pH exhibited a similar trend to the dechlorination performance. Cell abundance peaked at $5.6\pm0.4 \times 10^7$ cells/ml when 0.45 mM 1,2-DCA was converted to ethene by Day 4, with a growth yield of $1.3\pm0.08 \times 10^5$ cells/μM DCA removed at pH 7.0 (Figure 1c & 1d). Subsequently, cell abundance showed a decreasing trend as pH declined from 7.0 to 5.0, with a growth yield being 3.6 times lower at $3.5\pm0.3 \times 10^4$ cells/μM DCA removed (Figure 1c & 1d). Accordingly, the cell doubling time varied by an order of magnitude, with the fastest doubling time being 0.91±0.08 day and the slowest being 9.24±0.48 days (Figure 1d).

Genomic phylogenetic analysis identified strain AusDCA as a novel species within *Desulfitobacterium* genus

The genome of strain AusDCA was assembled using a hybrid assembly approach incorporating PacBio CLF and Illumina Novaseq reads. The final assembly yielded a 4,540 kbp chromosome, with a GC content of 41.7%, encoding 4,493 coding sequences, 79 tRNA and six rRNA operons. Based on 16S rRNA gene analysis, strain AusDCA was placed within the genus *Desulfitobacterium*, showing the highest sequence similarity (95.9%) to *Desulfitobacterium metallireducens* strain DSM15288. However, it showed lower similarity (92.5%–94.8%) to other members of *Desulfitobacterium* and to representatives from the genera *Acididesulfobacillus* and *Desulfosporosinus* in the family *Peptococcaceae* (Figure 2a).

Consistent with the 16S rRNA analysis, GTDB-Tk-based phylogenomic classification positioned strain AusDCA as a distinct and divergent lineage within the class-level reference tree. It shared an average nucleotide identity (ANI) of only 75.0%-well below the commonly accepted species-level threshold of 95-96% [43]- and a placement fraction of 0.28 relative to its closest reference genome, *D. metallireducens* DSM15288. The genome showed high

completeness, with 95.85% of marker proteins aligned in the multiple sequence alignment (MSA), and a Relative Evolutionary Divergence (RED) value of 0.90, indicating substantial evolutionary distance from known taxa. Analysis of the percentage of conserved proteins (POCP) further supported this distinction, with strain AusDCA sharing 61.7% POCP with *D. metallireducens*. While ANI and POCP values with other *Desulfitobacterium* species and related genera were below 70% and 55%, respectively—both below commonly accepted genus-level thresholds, which further support the classification of strain AusDCA as a novel lineage (Figure 2b, Figure S1&S2).

Despite its distant relationship to most members of the *Desulfitobacterium* genus, the relatively higher similarity to *D. metallireducens* DSM15288 supports the placement of strain AusDCA within this genus. Taken together, these findings suggest that strain AusDCA represents a novel acidotolerant organohalide-respiring bacterium (OHRB) within the genus *Desulfitobacterium*. We tentatively name this new species *Desulfitobacterium aciditolerans* sp. nov., with strain AusDCA as the type strain, pending comprehensive chemotaxonomic characterization.

Effects of acidic environment on proteins involved in organohalide respiration by strain AusDCA

Non-metric multidimensional scaling (NMDS) analysis elucidated distinct proteomic profiles under varying pH conditions (ANOSIM, R=1, p=0.03; Figure 3a), thereby indicating that the metabolic activities in strain AusDCA are markedly influenced by acidic environments. Out of the 4,493 predicted coding sequences in AusDCA, a total of 1,361 proteins were consistently detected under both pH conditions. The average number of proteins identified was 1,163 ± 38 at pH 7.0 and 1,223 ± 120 at pH 5.5, revealing no significant difference in protein

yield between the two conditions (t test, $p = 0.37$). Of these, approximately 20.4% (277 proteins) exhibited significant differential production (t-test, $|\log2|>1.5$, $p<0.05$), with 167 proteins upregulated and 90 proteins downregulated (Figure 3b, Table S1&S2).

We specifically investigated proteins involved in three key metabolic activities related to organohalide respiration: electron generation, electron transport, and electron consumption during the dehalogenation process (Figure 4a). In strain AusDCA, these metabolic processes exhibited distinct alterations under acidic conditions. As the key module in dehalogenation process, 1,2-DCA dechlorination, coupled with electron consumption, was mediated by the reductive dehalogenase operon, which was composed of four enzymes (AusDCA_4037-4040), the RDase catalytic subunit (DcaA), the RDase anchoring protein (DcaB), NosR type transcriptional regulator (DcaC), and putative trigger factor (DcaT) (Figure 4b). Three of these were produced, DcaA, DcaB and DcaT, with their abundance varying by three orders of magnitude in the range of $6.1\pm4.9 \times 10^5$ (DcaB) to $1.2\pm0.2 \times 10^8$ (DcaA) at pH 5.5, showcasing an upregulation of 1.5-3.0 folds ($p<0.05$) under acidic condition (Figure 4c).

In contrast, organohalide respiration associated with electron production were downregulated under acidic conditions. Strain AusDCA can generate electrons either via hydrogen uptake, a process involving the oxidation of H_2 by hydrogenase, or through pyruvate metabolism, where pyruvate serves as both a carbon source and an electron donor through pyruvate synthase. At pH 5.5, production levels of hydrogenase large and small unit decreased by approximately half, to $1.4\pm0.4 \times 10^7$ and $9.7\pm5.8 \times 10^5$, respectively. In pyruvate metabolism, the pyruvate synthase production dropped 28-fold to $3.1\pm0.9 \times 10^5$ (Figure 4c), indicating a strong downregulation of this primary NADH-generating and carbon entry point to the TCA cycle. Despite the reduced expression of these upstream NADH-producing enzymes involved in

1,2-DCA dechlorination, NADH-quinone oxidoreductase, which directly catalysed the electron transfer from NADH to quinone and was further utilized in 1,2-DCA dechlorination, was significantly upregulated. This may reflect a shift in cellular redox strategy under acid stress, whereby limited NADH—potentially derived from alternative metabolic sources such as residual glycolytic flux, amino acid metabolism, or other dehydrogenase reactions—is funneled efficiently into the respiratory chain. Rather than supporting high metabolic throughput, the upregulation of NADH-quinone oxidoreductase likely contributes to maintaining proton motive force and intracellular pH homeostasis by maximizing energy conservation from available reducing equivalents. Furthermore, the downregulation of pyruvate synthase also suggests suppressed entry into the TCA cycle via acetyl-CoA, consistent with a broader reduction in central metabolic activity. Meanwhile, the production of downstream electron transfer components such as b-type cytochromes and menaquinone biosynthesis protein remained unchanged (p>0.05), indicating a preserved capacity for electron transport, despite reduced input from primary electron-generating pathways. (Figure 4c).

Consumption of excessive cytoplasmic protons dominated the intracellular pH homeostasis

The maintenance of intracellular pH in strain AusDCA primarily relies on two major mechanisms: transmembrane proton transport and the consumption of excessive cytoplasmic protons. Proteomic profiling has identified seven key metabolic activities contributing significantly to pH regulation, with six of these activities associated with proton consumption, indicating this as the predominant mechanism. Among the differentially produced proteins, decarboxylases play a critical role by removing carboxyl groups from organic acids, producing neutral or basic compounds and reducing intracellular acidity [44]. Two decarboxylases (AusDCA_0751, 0754) were notably abundant in acidic conditions, produced at $3.24 \pm 0.26 \times 10^8$

and $1.37\pm0.15 \times 10^7$, which is 3.3 and 3.2 times higher compared to neutral pH, underscoring their importance in stabilizing intracellular pH. These two decarboxylases in strain AusDCA are uniquely present within the *Desulfitobacterium* genus, with no significant similarity detected in closely related *Desulfitobacterium* genomes (Figure S3). This unique feature may explain why strain AusDCA is currently the only identified acidotolerant strain within the *Desulfitobacterium* genus. Secondly, in nitrogen cycling, dissimilatory nitrate reduction to ammonia, which involves two stepwise reductive processes, nitrate to nitrite and continuous conversion from nitrite to ammonia and nitrogen fixation both contribute to proton consumption[45, 46]. Proteins involved in these processes (AusDCA_4263-4267) were significantly upregulated, with nitrogenase-related biosynthetic machinery upregulated 3.3 to 8.6 times higher, ranging from $1.11\pm0.30 \times 10^6$ to $2.24\pm0.50 \times 10^7$. Thirdly, intracellular pH can be adjusted by accepting protons via molecules with amino groups. Ethanolamine, derived from the breakdown and utilization of phospholipids, serves multiple roles in bacterial metabolism and provides metabolic flexibility[47, 48]. In strain AusDCA, proteins involved in the conversion of ethanolamine to acetate and ethanol (AusDCA_3707-3714) were dramatically downregulated under acidic conditions, particularly the ethanolamine ammonia lyase (EutBC, AusDCA_3710), produced 153.8 times lower under acidic conditions, decreasing from $1.73\pm0.74 \times 10^7$ to $1.12\pm0.83 \times 10^5$. Proteomic analysis revealed upregulation of biosynthesis of pathways for basic amino acids such as arginine (AusDCA_3520-3534) and histidine (AusDCA_2789-2793) [49], whose side chains contain high numbers of amino ($-NH_2$) groups. These pathways were significantly upregulated at pH 5.5, with protein production levels increasing by 2.9- to 22.1-fold compared to neutral conditions. This buffering action helps to stabilize the intracellular environment, preventing detrimental fluctuations in pH that affect cellular functions. Additionally, enhanced nucleotide synthesis for

pyrimidine and purine biosynthesis also plays a crucial role, facilitating DNA and RNA repair, with genes upregulated up to 19.0 times higher [50, 51]. Furthermore, aldehyde dehydrogenase (ALDH, AusDCA_1531), upregulated 12.6 times to $1.23\pm0.38 \times 10^7$, mitigates oxidative stress by removing toxic aldehydes, contributing to cell survival under acid stress [52]. Lastly, proton pumping via NADH-quinone oxidoreductase helps maintain the proton motive force (PMF) essential for ATP synthesis and cellular processes [53]. Corresponding protein expression was markedly upregulated under acidic conditions, with NuoL increasing 2.8-fold to $1.03\pm0.30 \times 10^5$, NuoH rising from below detection to $7.27\pm2.65 \times 10^5$, and NuoF increasing 7.1-fold to $1.17\pm0.13 \times 10^6$, underscoring the importance of this complex in acid stress adaptation.

Energy conservation strategies for acid stress adaptation

Acid stress can significantly impact energy utilization in bacteria, disrupting normal cellular processes and forcing the cells to adapt their energy metabolism to cope with the challenging conditions. In strain AusDCA, three metabolic activities, nicotinate fermentation, 3-hydroxybutyrate (3-HB) production, and flagellation, have been primarily discovered participating in acid stress adaptation from the perspective of energy flow [54]. Nicotinate (niacin, vitamin B3) is a crucial component in all living cells, primarily existing as nicotinamide adenine dinucleotide (phosphate) [NAD(P)]. Cells maintain NAD(P) concentrations between 0.1 and 1 mM, providing nicotinate as a source of nitrogen, carbon, and energy to a diverse range of microorganisms that specialize in nicotinate catabolism[55]. Proteins (AusDCA_4162, 4165-4171) involved in the catabolism from nicotinate to pyruvate have been upregulated ranging from 3.7 to 15.2 times. The conversion of nicotinate to pyruvate generates intermediates that feed into central metabolic pathways such as glycolysis and the TCA cycle. This ensures a steady supply of ATP, which is crucial for powering various cellular processes, including those involved in pH

homeostasis. While the production of 3-HB, often as a monomer of polyhydroxybutyrate (PHB), is a well-documented response to various stresses and a method for carbon and energy storage [56]. Under acid stress conditions, a downregulation of proteins associated with 3-HB production (AusDCA_3498-3501) was observed, showing a significant decrease in protein production ranging from 3.0 to 6.0 times (p<0.05). While direct metabolite measurements were not conducted, this translational downregulation, along with upregulation of nicotinate fermentation pathways, suggests a potential shift in pyruvate utilization. These changes may indicate a redirection of carbon flux toward the TCA cycle rather than 3-HB biosynthesis, possibly as an adaptive response to maintain energy production under acidic conditions. Additionally, strain AusDCA downregulated flagellation modulars, including the production and activity of flagella, as part of their adaptive response (AusDCA_0322, 0324, 0325, 0330, 0351, 0377, 0378, 0381). Flagella motility is an energy-intensive process. The synthesis, assembly, and operation of flagella require significant amounts of ATP. Under acid stress, conserving energy becomes critical, and downregulating flagellation allows the cell to redirect energy towards essential stress response mechanisms and maintenance of pH homeostasis. Overall, the gene operon for flagellation exhibited the downregulation, at the largest extent among all the affected metabolic pathway under acid stress, ranging from 5.1-67.5 times decrease and averagely at 24.8 times. Taken together, the combined effects lead to a significant reallocation of energy resources, by altering metabolic pathways to more efficient modes of energy utilization, increasing the need for cellular repair and maintenance, inhibiting enzyme activity, and triggering energy-consuming stress responses.

DISCUSSION

Acidic conditions significantly impact the kinetics and pathways of biochemical reactions involved in organohalide respiration. Understanding the specific adaptations that enable survival and function in low pH environments provides critical insights into the metabolic and regulatory networks of organohalide-respiring bacteria (OHRB). In this study, we investigated the influence of acidic environments on organohalide respiration activities and adaptation mechanisms in strain AusDCA, identified as a novel acidotolerant organohalide-respiring bacterium (OHRB) within the genus *Desulfitobacterium*. Our findings revealed that acidic conditions downregulate the assimilatory metabolism of pyruvate, mediated by pyruvate synthase, which slows core metabolic activities and results in decreased growth rates. To compensate, the reductive dehalogenase operon was upregulated, maintaining dehalogenation activities despite the adverse conditions. As a facultative OHRB, strain AusDCA can acquire electron for reductive dehalogenation either through hydrogen uptake via hydrogenase or from intracellular metabolic processes. In this study, although pyruvate was supplied as the sole external electron donor, it is likely that intracellular hydrogen produced during pyruvate oxidation was re-utilized via hydrogen cycling. The detection of hydrogenases under neutral conditions support this possibility, indicating that hydrogen cycling may play a role in redox balancing and energy conservation during anaerobic metabolism. However, under acidic conditions (pH 5.5), a marked decrease in hydrogenase expression was observed, suggesting a diminished role for hydrogen cycling. This downregulation implied a metabolic shift toward more direct electron transfer mechanisms, potentially mediated via NADH oxidation pathways. Such a shift may reflect an adaptive response strategy to low pH environments, where the altered redox potential and increased proton concentrations may render hydrogen-based electron transfer less favorable.

Moreover, the apparent preference for NADH-to-NAD^+ conversion as a source of reducing equivalents may offer the additional benefit of intracellular proton consumption or expulsion, aiding in pH homeostasis. This shift in electron transfer strategy under acidic conditions highlights an important consideration for *in situ* bioremediation: selective stimulation of metabolic pathways that favor direct electron donors like pyruvate or other reduced carbon sources may enhance efficiency and reduce cost, particularly in acidic environments.

Strain AusDCA exhibits a robust and distinctive set of adaptive strategies that enable survival and function in acidic environments, underscoring its potential as a model organism for understanding microbial life under extreme pH stress. Proteomic profiling revealed that AusDCA primarily employs proton consumption and transmembrane proton transport to regulate intracellular pH. The predominance of proton-consuming metabolic pathways distinguishes AusDCA from other acid-tolerant or acidophilic relatives and highlights a potentially more energetically favorable mechanism for acid stress mitigation. Unlike *Acididesulfobacillus acetoxydans*, which can survive at pH 3.8, by relying heavily on ion transport systems (e.g., proton efflux and alkali metal ion uptake) and increased expression of molecular chaperones, AusDCA adopts a metabolically focused strategy. Similarly, *Desulfosporosinus* spp., which utilize membrane-bound proton pumps and synthesize extracellular polymeric substances (EPS) to resist acid stress, exhibit a more structurally oriented approach. In contrast, AusDCA relies on internal metabolic buffering reactions, indicating a distinct biochemical strategy that effectively supports cellular homeostasis with reduced structural or energetic burden. This unique physiological profile positions AusDCA as a compelling example of evolutionary innovation in acid stress adaptation. Its reliance on metabolic proton consumption suggests a more sustainable and potentially versatile mechanism for acid tolerance, offering a valuable contrast to the ion-

and structure-based strategies observed in other taxa. Such insights enhance our broader understanding of microbial resilience in extreme environments and underscore the diversity of evolutionary solutions to pH stress. Further, The availability of genomic and proteomic data further enables future comparative studies to identify conserved and unique acid-tolerance traits among organohalide-respiring bacteria.

By elucidating the mechanisms underlying novel strain AusDCA's acid tolerance, this study contributes to a deeper understanding of microbial adaptation and physiology under acidic stress. The distinctive strategies employed by AusDCA expand the conceptual framework of acidophilic and acidotolerant microbial life and offer promising directions for future research into low-pH microbial ecology and biotechnology.

ASSOCIATED CONTENT

Supporting Information

More details about genomic characterization and proteomic data of strain AusDCA are provided in supporting information.

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FIGURE LEGENDS

Figure 1. Dechlorination performance of strain AusDCA on 1,2-DCA. (a) Dechlorination kinetics, (b) dechlorination rates, (c) cell abundance, estimated by dividing 16S rRNA gene copy number by six, and (d) growth yield and doubling time across a pH range of 7.5 to 4.5.

Figure 2. Phylogeny of strain AusDCA inferred by (a) 16S rRNA gene analysis and (b) Average Nucleotide Identity (ANI) with its closest neighboring members from Peptococcaceae family (i.e., *Desulfosporosinus*, *Desulfitobacterium*, and *Acididesulfobacillus*). ▲: the 16S rRNA gene copies from strain AusDCA; *: the 16S rRNA gene copy with a 164 bp insertion sequence; ●: the strain identified as the closest phylogenetic relative to strain AusDCA.

Figure 3. Differential proteomic profiles of strain AusDCA at pH 5.5 and pH 7.0. (a) NMDS analysis and (b) Volcanic plot (|fold change| > 1.5, $p < 0.05$).

Figure 4. Schematic diagram of (a) 1,2-DCA respiration (H_2 is produced from pyruvate fermentation process), (b) reductive dehalogenase operon in strain AusDCA, and (c) effects of pH on proteins involved in 1,2-DCA respiration.

Figure 5. Multiple mechanisms contribute to the adaption mechanisms to acidic environment. (a) Schematic representation of all the mechanisms of protection against acid stress that occur in strain AusDCA. Actively expelling protons out of the cell via (i) decarboxylation; (ii) H+ consumption in nitrogen cycling; (iii) less ethanolamine consumption; (iv) enhancement of synthesis of basic amino acids; macromolecules protection and repair in (v) pyridine and purine biosynthesis and (vi) aldehyde dehydrogenase; (vii) proton cycling in NADH-quinone electron transport chain; (b) differentially expressed genes involved in acid defense pathways.

Figure 6. Energy conservation mechanisms under acid conditions. (a) Metabolic pathways of upregulation of nicotinate fermentation and downregulation of 3-HB production; (b)

differentially expressed genes involved in (i) nicotinate fermentation, (ii) 3-HB production, and (iii) flagella assembly and motivation.

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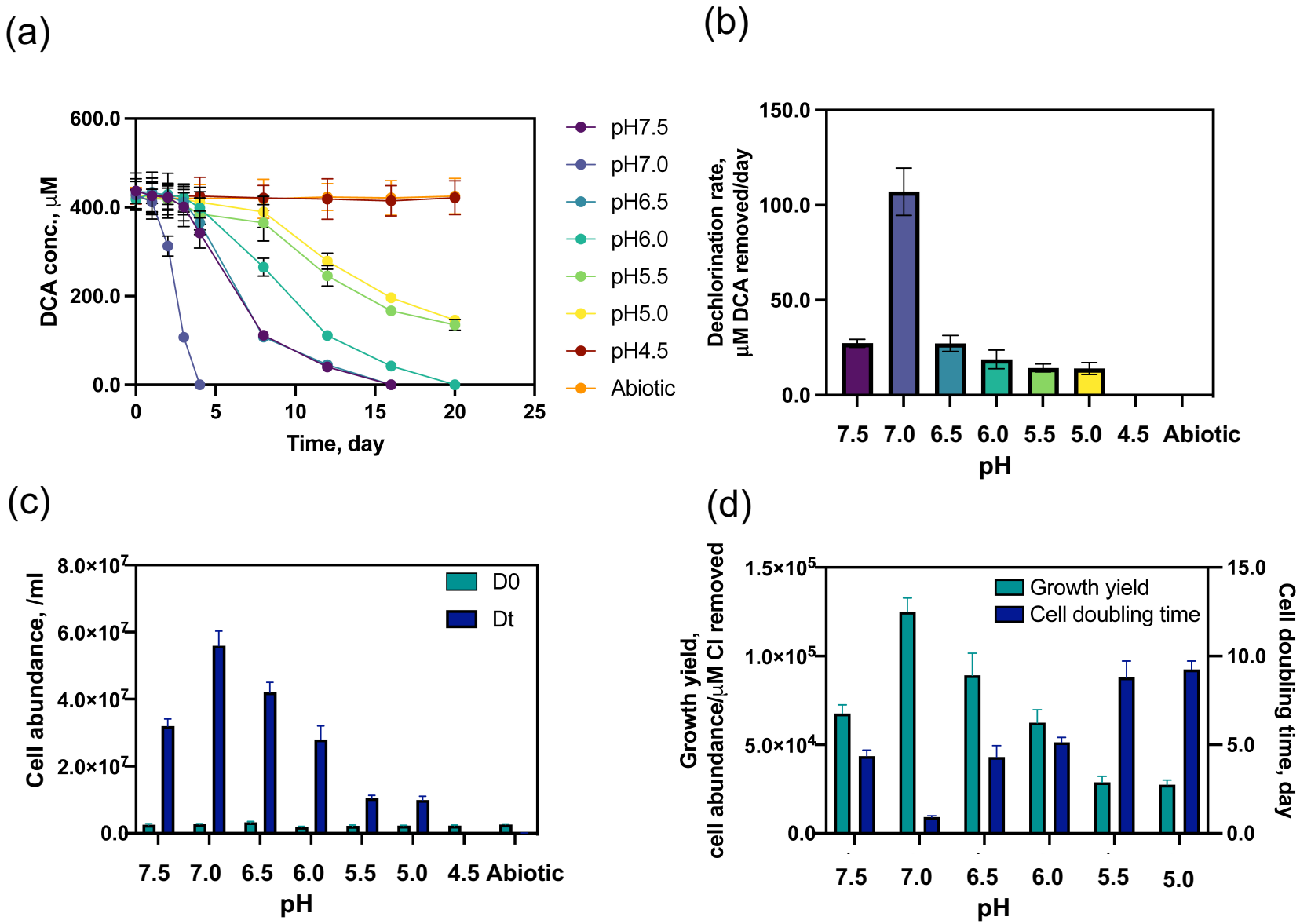

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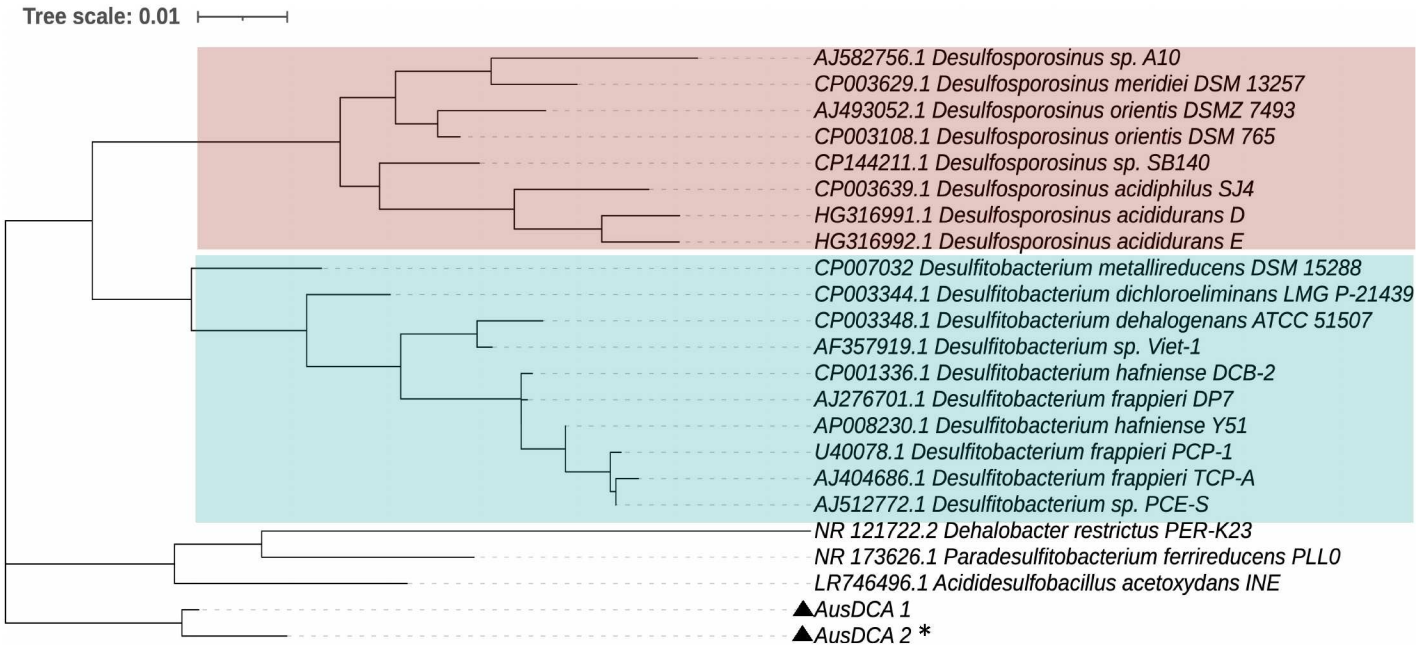
(a)



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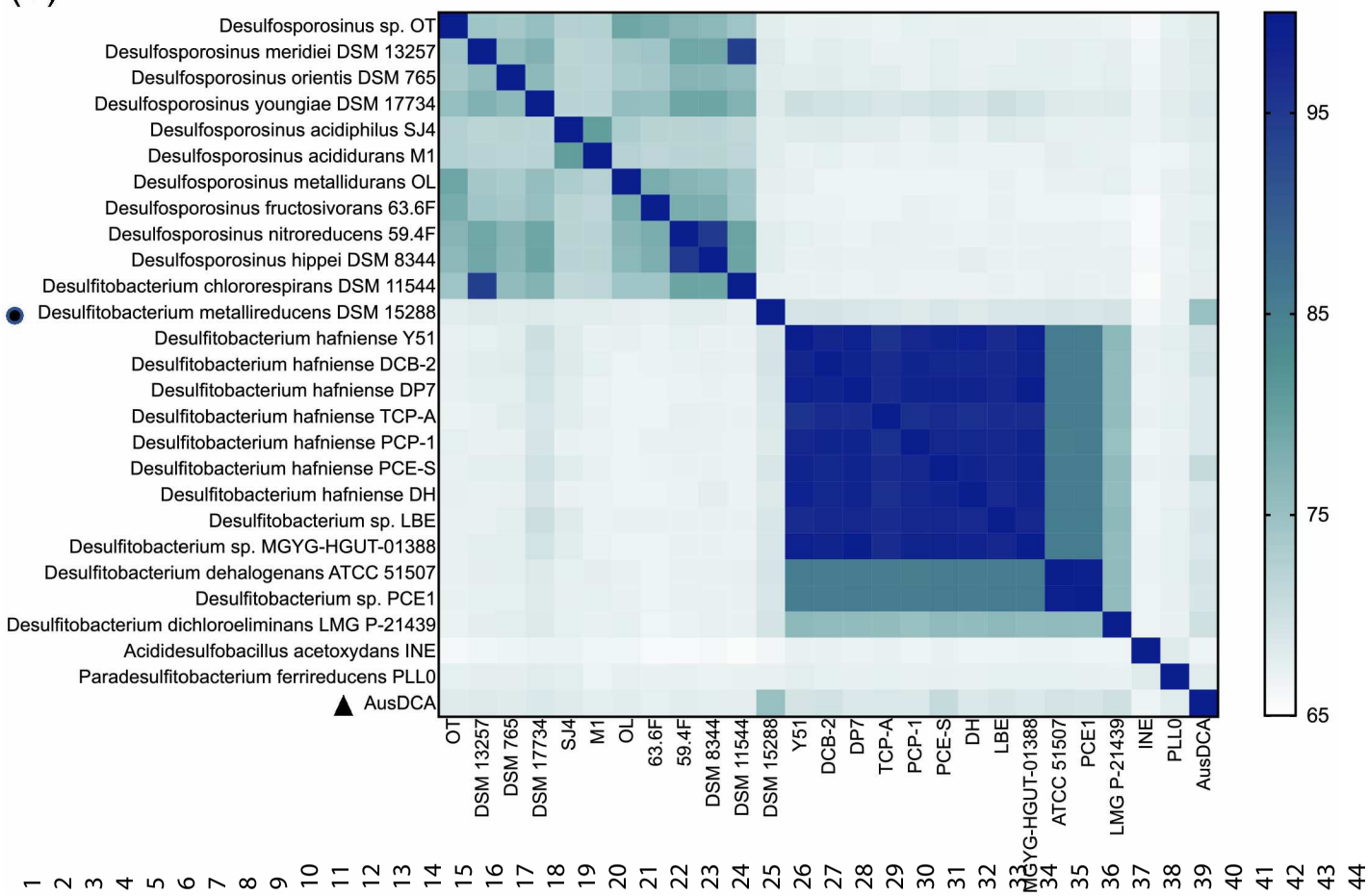

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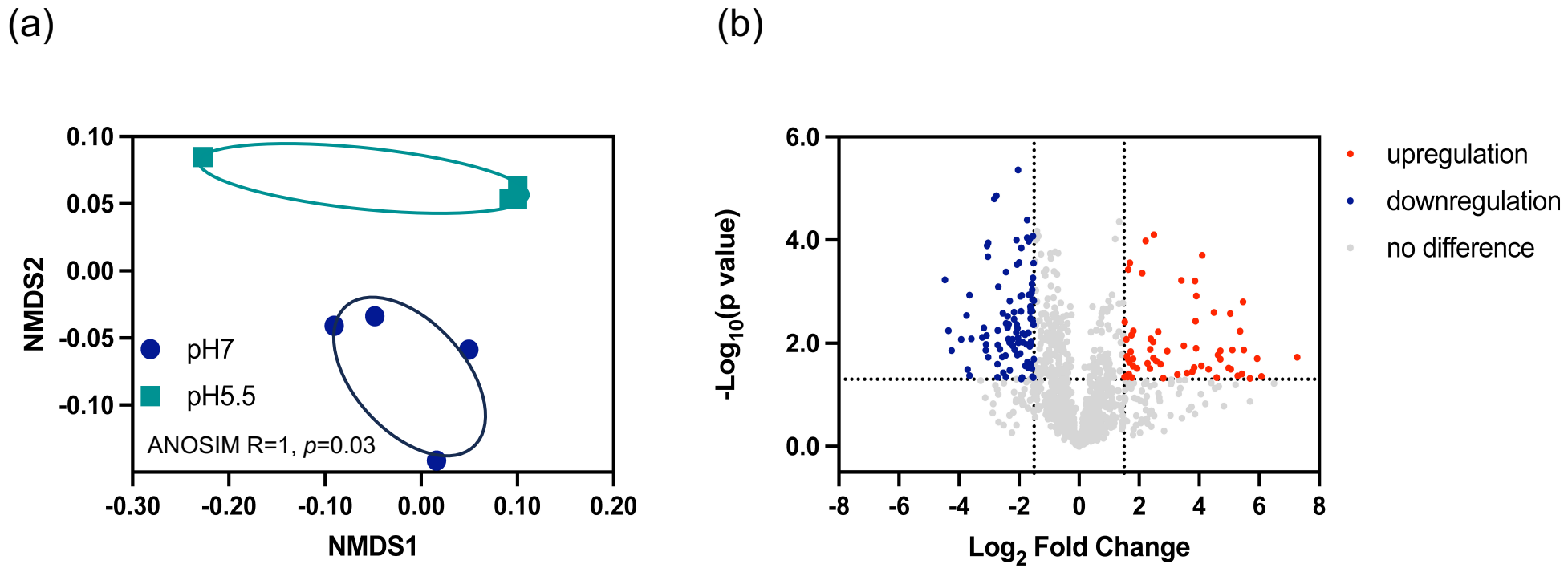

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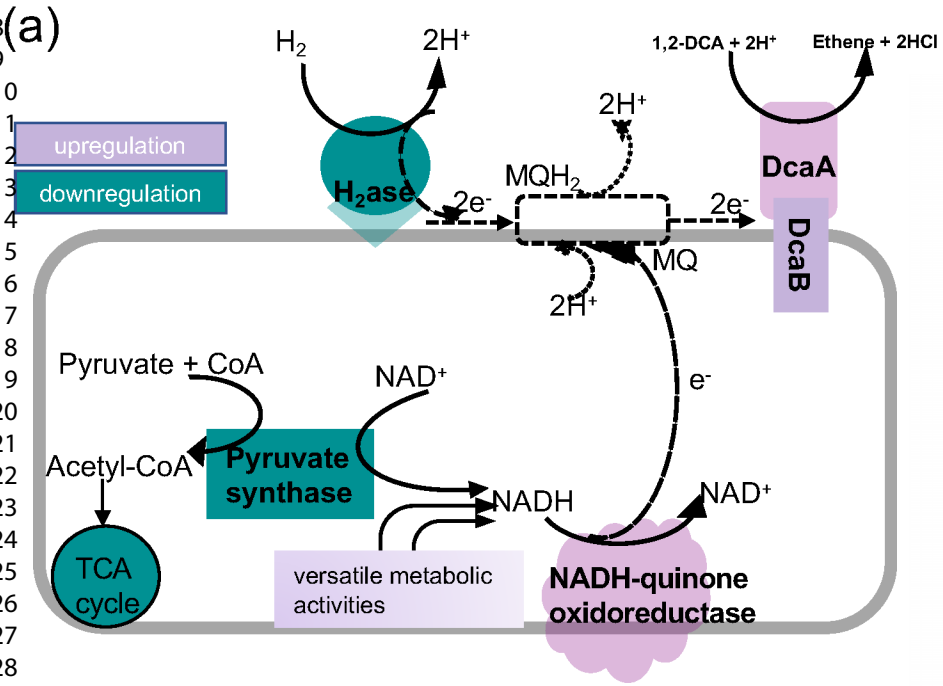
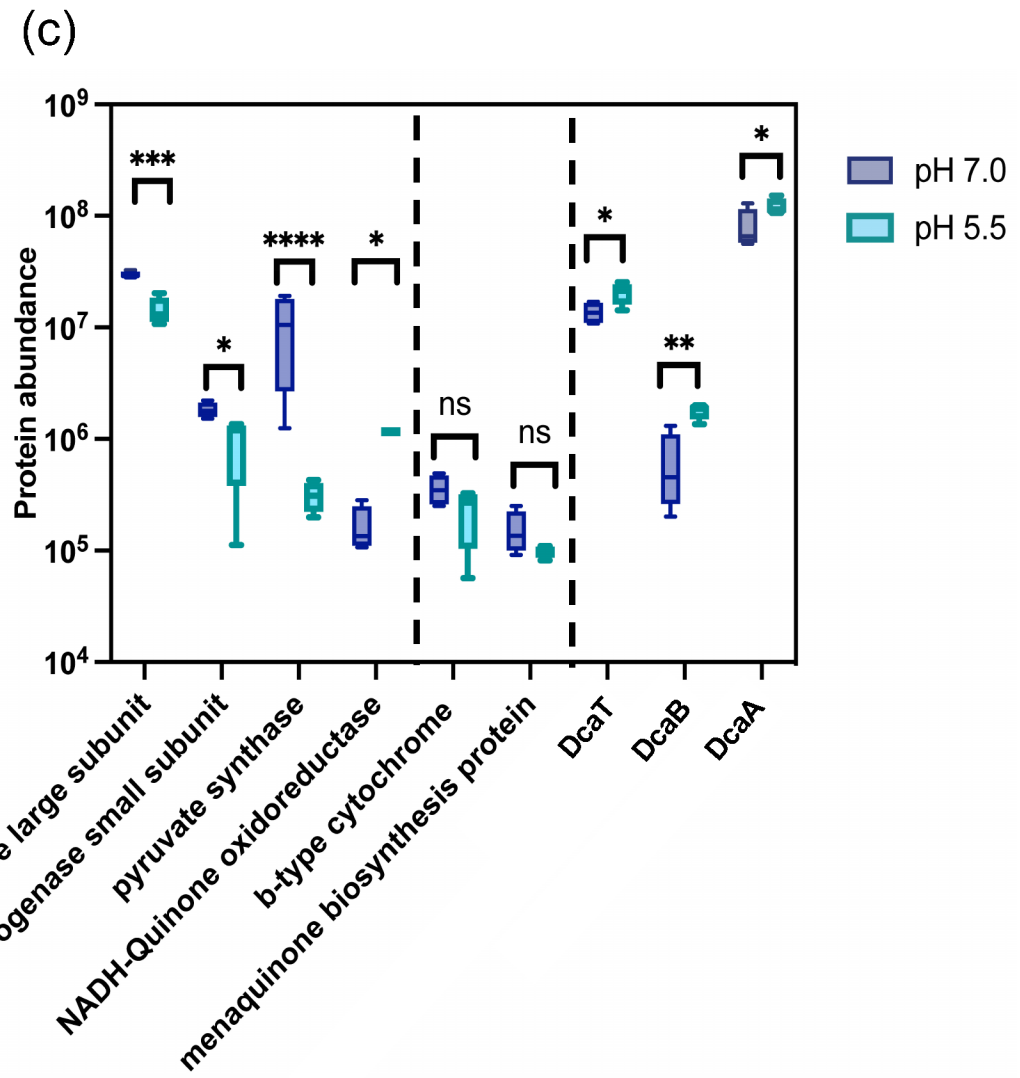


dcaA: DCA RDase catalytic subunit
dcaB: DCA RDase anchoring protein
dcaC: NosR type transcriptional regulator
dcaT: putative trigger factor



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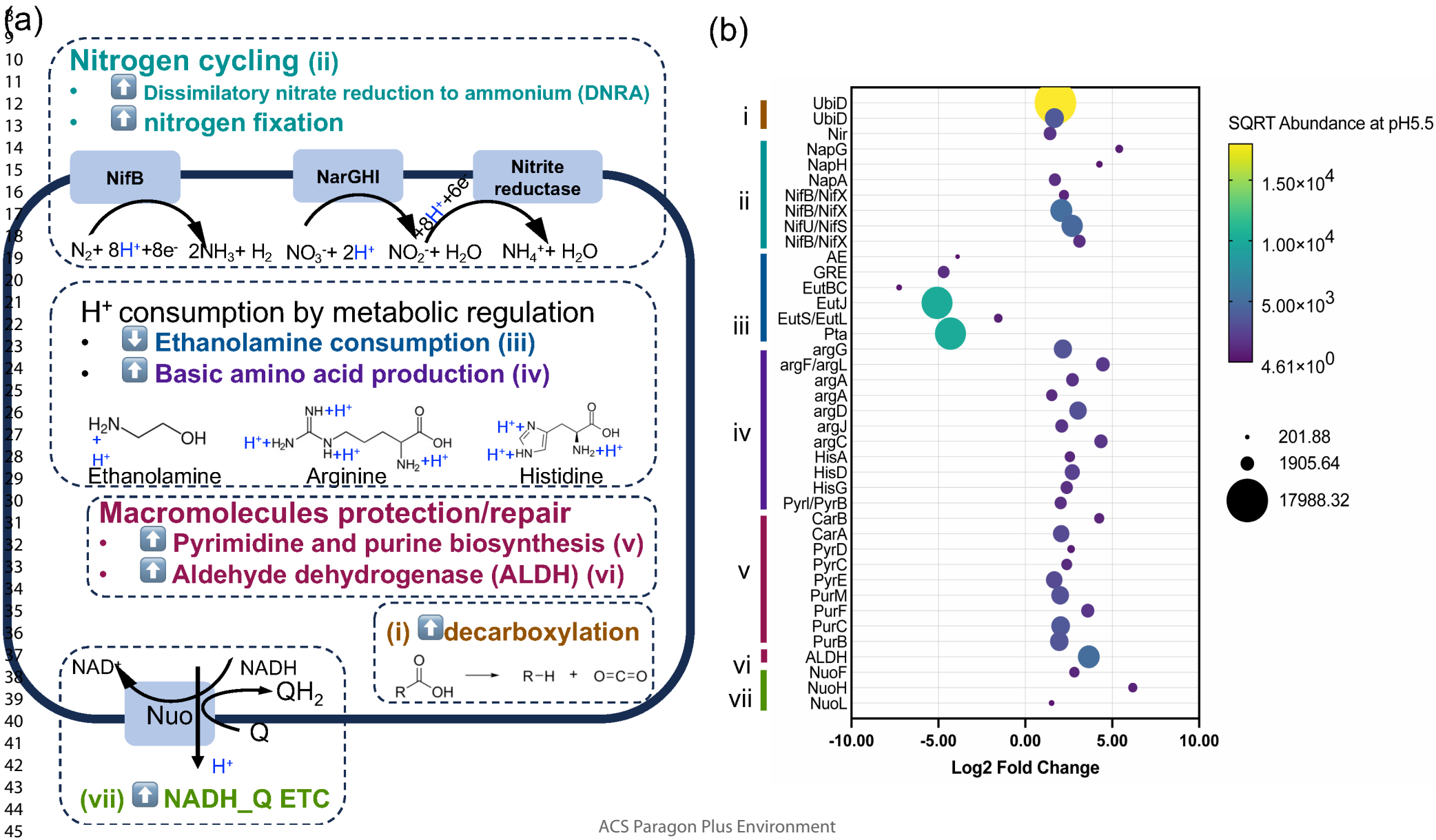

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