

Genome-Wide Responses of the Model Archaeon *Halobacterium* sp. Strain NRC-1 to Oxygen Limitation

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As part of a comprehensive postgenomic investigation of the model archaeon *Halobacterium* sp. strain NRC-1, we used whole-genome DNA microarrays to compare transcriptional profiles of cells grown under anaerobic or aerobic conditions. When anaerobic growth supported by arginine fermentation was compared to aerobic growth, genes for arginine fermentation (*arc*) and anaerobic respiration (*dms*), using trimethylamine *N*-oxide (TMAO) as the terminal electron acceptor, were highly upregulated, as was the *bop* gene, required for phototrophic growth. When arginine fermentation was compared to anaerobic respiration with TMAO, the *arc* and *dms* genes were both induced with arginine, while TMAO induced the *bop* gene and major gas vesicle protein (*gvpAC*) genes specifying buoyant gas vesicles. Anaerobic conditions with either TMAO or arginine also upregulated the *cba* genes, encoding one of three cytochrome oxidases. In-frame deletion of two COG3413 family regulatory genes, *bat* and *dmsR*, showed downregulation of the *bop* gene cluster and loss of purple membrane synthesis and downregulation of the *dms* operon and loss of anaerobic respiration capability, respectively. Bioinformatic analysis identified additional regulatory and sensor genes that are likely involved in the full range of cellular responses to oxygen limitation. Our results show that the *Halobacterium* sp. has evolved a carefully orchestrated set of responses to oxygen limitation. As conditions become more reducing, cells progressively increase buoyancy, as well as capabilities for phototrophy, scavenging of molecular oxygen, anaerobic respiration, and fermentation.

The physiological versatility of halophilic *Archaea* (*Haloarchaea*) has been of interest since the recognition of the three domains of life (15). Unlike most other *Archaea*, which are strict anaerobes, *Haloarchaea* grow best aerobically yet can generate metabolic energy also via anaerobic respiration, fermentation, and photophosphorylation. Among the *Haloarchaea*, *Halobacterium* sp. strain NRC-1 is a genetically tractable model organism with a 2.0-Mb chromosome and two dynamic megaplasmids or minichromosomes, pNRC100 (191 kb) and pNRC200 (365 kb) (11, 24, 25). Analysis of the genome sequence identified ~2,500 genes, and postgenomic studies showed many to be responsive to fluctuations in environmental conditions (12). *Halobacterium* sp. NRC-1 is highly responsive to various oxygen levels, including microaerobic and anaerobic conditions, fluctuating levels of salinity to near saturation, and a wide range of temperatures from -20°C to 60°C (9). The organism has also been shown to be tolerant of high levels of UV and ionizing radiation, which is an adaptation to the high solar radiance and desiccating conditions in its environment (16, 21). As a result, *Halobacterium* sp. NRC-1 has been valuable for studies of multiple stress responses in the *Archaea* (30).

Although *Halobacterium* sp. NRC-1 grows fastest under aerobic conditions, it has been shown to have the capacity for anaerobic growth via substrate-level phosphorylation using arginine, by anaerobic respiration using dimethyl sulfoxide (DMSO) or trimethylamine *N*-oxide (TMAO), as well as phototrophic growth using the light-driven proton pump in its purple membrane (1, 11, 23, 28, 31). Anaerobic fermentation occurs via the arginine deiminase (ADI) pathway, which is encoded by the *arcRACB* genes (25, 28), while anaerobic respiration requires a DMSO/TMAO reductase and a chaperone, encoded by the *dmsREABCD* operon. Light-driven proton pumping utilizes bacteriorhodopsin, a retinal chromoprotein that forms a two-dimensional array in the

cell membrane and that is encoded by the *bop* gene cluster. In contrast to the *arc* genes, which are located on pNRC200, both the *dms* operon and *bop* gene cluster are encoded on the *Halobacterium* chromosome (1, 23, 25).

A combination of pre- and postgenomic studies has established basic genetic features of purple membrane biosynthesis and phototrophy in *Halobacterium* sp. NRC-1. The protein component, bacterio-opsin, is the product of the *bop* gene, while the chromophore retinal requires *crtB1* and *brp*, which specify the first and last committed steps in the retinal biosynthetic pathway, respectively (1, 27). These genes are clustered together with six additional genes: (i) a sensor regulator gene, *bat*; (ii and iii) two small genes, *brz* and *brb*, thought to be involved in regulation; (iv) *bac*, encoding a putative AAA⁺ ATPase chaperone; (v) *bap*, encoding a putative membrane protein of unknown function downstream of *bop*; and (vi) *blp*, encoding a soluble protein of unknown function (14, 25, 27, 29, 32, 33). The *bat* gene codes for a multidomain protein with a putative redox-sensing PAS-PAC or LOV (COG2202) domain and a light-sensing GAF domain (COG2203), as well as a C-terminal helix-turn-helix (HTH) DNA binding domain (COG3413) (1, 26). Extensive analysis of *Halobacterium* mutants lacking purple membrane implicated the *bat* gene product in regulation of several genes in the *bop* gene

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cluster, and saturation mutagenesis of the *bop* promoter region identified an upstream activator sequence (UAS) as the regulatory site of action (1, 4). Bioinformatic analysis also revealed the presence of a UAS upstream of the *brp*, *crtB1*, and *blp* genes, in addition to the *bop* gene, and these genes were shown to be induced under limiting oxygen conditions and to support phototrophic growth (1, 36). Coordinate induction of the *bop* gene and the major gas vesicle protein gene, *gvpA*, under microaerobic conditions was also reported (36). Therefore, gas vesicle production is also induced, increasing cell buoyancy and enhancing the availability of light and oxygen for photophosphorylation and oxidative phosphorylation.

In another postgenomic investigation, a custom *Halobacterium* sp. NRC-1 oligonucleotide microarray was used for transcriptional profiling to study the response of this model organism to anaerobic growth with either DMSO or TMAO (23). When cell growth was promoted by anaerobic respiration using either compound as the sole terminal electron acceptor, the *dmsREABCD* operon was found to be highly induced and essential. Deletion of the putative regulatory gene, *dmsR*, showed that utilization of these alternate terminal electron acceptors is dependent on this gene, consistent with its action as a transcriptional activator. The C-terminal region of the *dmsR* gene product was shown to contain an HTH DNA binding motif (COG3413) similar to that of the *bop* gene activator protein, Bat, although no LOV or GAF domain was found to be present. The *cbadBA* genes, encoding a *ba₃*-type cytochrome oxidase, were also found to be inducible when grown with TMAO (23). The *cbmA* and *cbmB* genes code for the cytochrome oxidase subunits, likely functioning at low oxygen partial pressure, while *cbmD* codes for an additional small hydrophobic protein (20).

In the present study, we have used transcriptomic, mutational, and bioinformatic analyses to advance our understanding of the response of *Halobacterium* sp. NRC-1 to anaerobic conditions. Our results show that a complex genetic program involving multiple regulatory genes orchestrates the response of *Halobacterium* to changes in oxygen availability and that cells stay primed for aerobic respiration even under anaerobic conditions.

MATERIALS AND METHODS

Culturing. *Halobacterium* sp. NRC-1 (Table 1) was grown aerobically or anaerobically with TMAO as described previously (13, 23). For fermentative growth, arginine was added to a final concentration of 0.5% (wt/vol) to modified complex (CM⁺) medium containing 0.001 g/liter peptone and stored inside an anaerobic glove box (Coy Laboratory Products) for 1 day prior to inoculation. Anaerobic conditions during fermentation were ascertained with resazurin as described previously (23). The cultures were grown in the dark at 37°C and 100 rpm for up to 8 days.

Deletion construction. The *bat* mutant was generated using the standard *Halobacterium* sp. NRC-1 deletion method (2), as reported previously for the *dmsR* mutant (23) (Table 1 and 2). Briefly, a suicide plasmid vector was created by triple ligation of ~500 bp of PCR-amplified flanking regions 5' and 3' of the *bat* gene in *Halobacterium* sp. NRC-1 into the multiple-cloning site of pBB400 (2), which contains a wild-type copy of the *Halobacterium* sp. NRC-1 *ura3* gene plus its native promoter, and transformed into *Escherichia coli* DH5 α (10). Cells were plated onto LB plates containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Ten colonies were picked, and plasmid preparations were done using the previously described method (2). The plasmids were checked using HindIII-XhoI double digests to release a 1-kb insert. One plasmid was used to transform *Halobacterium* sp. NRC-1 Δ *ura3* (Table 1) using the polyethylene glycol-EDTA method (2). Cells were plated

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source (reference)
<i>Escherichia coli</i> DH5 α		Laboratory collection (10)
<i>Halobacterium</i> spp.		
NRC-1	Wild type	Laboratory collection (2)
BB400	NRC-1 Δ <i>ura3</i>	Laboratory collection (2)
JAM101	BB400 Δ <i>dmsR</i>	Laboratory collection (23)
PAD101	BB400 Δ <i>bat</i>	This study
Plasmids		
pUC19	Cloning vector, <i>lacZ</i> α <i>bla</i>	New England Biolabs
pBB400	pUC19 containing NRC-1 <i>ura3</i>	Laboratory collection (2)
pPAD101	pBB400 containing truncated <i>bat</i> with flanking region	This study

on agar plates containing uracil dropout medium (HURA⁺) and grown at 42°C. DNA from individual colonies, purified as previously described (2, 15), was used as the template in PCRs to screen for suicide plasmid integration into genomic DNA. Two independent isolates were plated onto CM⁺ medium containing 250 μ g/ml of 5-fluoro-orotic acid (5-FOA) for counterselection. Candidate colonies were picked and grown in CM⁺ 5-FOA liquid medium. DNA was isolated and PCR amplified using flanking primers (Table 2). Two potential deletants were identified out of 17 candidates and further verified by PCR using both internal and flanking gene primers and by Sanger sequencing.

DNA microarrays. Whole-genome custom DNA oligonucleotide microarrays were fabricated by Agilent Corporation and hybridized using standard *Halobacterium* sp. NRC-1 methods (2, 23). Cultures were harvested at an optical density at 600 nm (OD₆₀₀) of 0.49 for the arginine and TMAO experiments, while the OD for the Bat experiment was 1.0 (35). RNA was isolated using spin columns (Agilent), after cell lysis and DNase (Invitrogen) treatment. Synthesis of cDNA was performed with SuperScript II RNase H reverse transcriptase (Invitrogen) using 500 ng of RNA and 2 pmol of primers from three cultures grown under identical conditions. RNA from 3 parallel cultures was pooled to minimize biological noise, and fluorescently labeled cDNA was prepared as previously described (23). The labeled cDNA targets were combined and hybridized in duplicate for 17 h at 65°C in the dark, washed, and scanned for the Cy3 and Cy5 fluorescent signals on an Agilent DNA microarray scanner (model G2565BA). Image processing was carried out using Agilent Feature Extraction software. Array data have been deposited in the GEO database (see below).

Bioinformatic analysis. Agilent microarray data were analyzed using GeneSpring software, version 11.5.1. Green and red signal intensities of the probes were imported using custom-created software. Quantile normalization (5) was performed, and baseline transformation to the median of all samples was applied to each probe to normalize the signal intensities of each probe across all samples to its median. Green processed signal intensities were grouped and labeled Cy3, while red processed signal intensities were grouped and labeled Cy5. A new gene-level experiment was created in which quantile normalization and baseline transformation were performed. Interpretation was created by grouping the data sets into two groups: Cy3 and Cy5. Fold change analysis was performed by selecting pairs of conditions with the pairing option defining Cy3 and Cy5 as two conditions. To determine the differentially expressed genes that were statistically significant, a *P* value of <0.05 and a fold change cutoff threshold of ≥ 2 were used. Local clusters of proteins developed using reciprocal blasting (7, 8) as well as the COG cluster at NCBI were utilized to identify conserved proteins and domains.

TABLE 2 Oligonucleotides used for *bat* deletion construction

Name	Sequence	Description
batK1F	CTT AAG CTT CCC ACG CCA CCA TCA TCC	Outside primer, flanking <i>bat</i> gene on 3' end, HindIII site
batK1R	CTT ACT AGT CTC GTC GGC GCG TTC TTC	Inside primer, flanking <i>bat</i> gene on 3' end, SpeI site
batK2F	CTT ACT AGT CTC GGA CTC GGT GTT CTG GAC	Inside primer, flanking <i>bat</i> gene on 5' end, SpeI site
batK2R	CTT CTC GAG CGG TGA CAT CGC AAC CCG	Outside primer, flanking <i>bat</i> gene on 5' end, XhoI site
BB1464_For	ATG ACG AGC GTC CAG AAC AC	<i>bat</i> gene start primer
BB1464_Rev	CGT CAC TCC TCG AAG AAC GC	<i>bat</i> gene stop primer
BLABBF	ATG AGT ATT CAA CAT TTC C	Forward primer for β -lactamase gene on pBB400
BLABBR	TTA CCA ATG CTT AAT CAG T	Reverse primer for β -lactamase gene on pBB400

Membrane analysis. Purple and red membrane preparations were isolated using sucrose cushion gradients as previously described (13). Briefly, cells were grown in 1 liter medium for 1 to 2 days into stationary phase ($OD_{600} = 1.0$) and spun down in a Sorvall RC-5B centrifuge at 6,000 rpm and 4°C for 10 min. The supernatant was removed, and cells were resuspended in 5 ml basal salts solution. The cell lysate was transferred into dialysis tubing and dialyzed against 5 liters of water at 4°C with 3 changes. The cell paste was then treated with 50 μ l DNase I (10 μ g/ μ l) and incubated at 37°C for 1 h with shaking at 180 rpm. This step was repeated, when necessary, to reduce viscosity. The sucrose gradient was prepared in 40 ml nitrocellulose centrifuge tubes by adding filter-sterilized sucrose dissolved in water to the bottom of the tube in the order 11 ml 30% sucrose, 11 ml 40% sucrose, 11 ml 50% sucrose, and 2 ml 60% sucrose; and 5 ml lysate was layered on top of the gradient. The gradient was spun using an SW28 swinging-bucket rotor in a Beckman L5 ultracentrifuge for 15.5 h at 24,000 rpm under vacuum at 18°C.

Microarray data accession number. Array data were deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo>) under series accession number GSE38374 (18).

RESULTS

Anaerobic growth on arginine versus aerobic growth. *Halobacterium* sp. NRC-1 was grown either aerobically in standard shaken cultures or anaerobically with 0.5% arginine. RNA was prepared from both aerobically and anaerobically grown cells, and comparative transcriptional profiling was conducted using whole-genome oligonucleotide microarrays. Several genes involved in generation of metabolic energy had significant changes in transcript levels under anaerobic versus aerobic conditions (Fig. 1A and 2,

white bars). Surprisingly, the *dms* genes involved in DMSO/TMAO respiration were the most highly induced during arginine fermentation, although neither substrate for the DMSO/TMAO reductase was present. The *arcACB* genes, which are involved in arginine fermentation via the ADI pathway, were also strongly induced under anaerobic growth on arginine. During growth on arginine, the third catabolic gene, *arcA*, was somewhat less induced than *arcC* and *arcB*, similar to a previous report (28). The bacterio-opsin-encoding gene *bop* was also strongly upregulated, while the downstream transcriptionally linked gene *bap* was also induced, but less strongly. The other genes in the *bop* gene cluster, *brp* and *crtB1*, were only slightly changed, while *blp* and *bat* were essentially unchanged with arginine. Interestingly, among the cytochrome oxidase genes, the *cbaD* and *cbaB* gene transcripts were significantly upregulated under arginine fermentation almost as highly as the *arc* genes, while the *cbaA* gene was slightly upregulated. Among the gas vesicle gene cluster, only the major gas vesicle protein genes *gvpA* and *gvpC* were upregulated, but not highly (Fig. 2). These results suggest that progressively more reducing conditions result in increasing transcriptional induction, in a gradient from *gvp* and *bop* to the *cba*, *dms*, and *arc* genes.

Anaerobic growth on arginine versus TMAO. Transcriptional analysis was carried out for *Halobacterium* sp. NRC-1 grown anaerobically with arginine fermentation versus anaerobic respiration using TMAO (Fig. 1B and 2, light gray bars). Among genes involved in metabolic energy generation, the *dmsEABC* and *arcACB* genes were induced during anaerobic growth on arginine

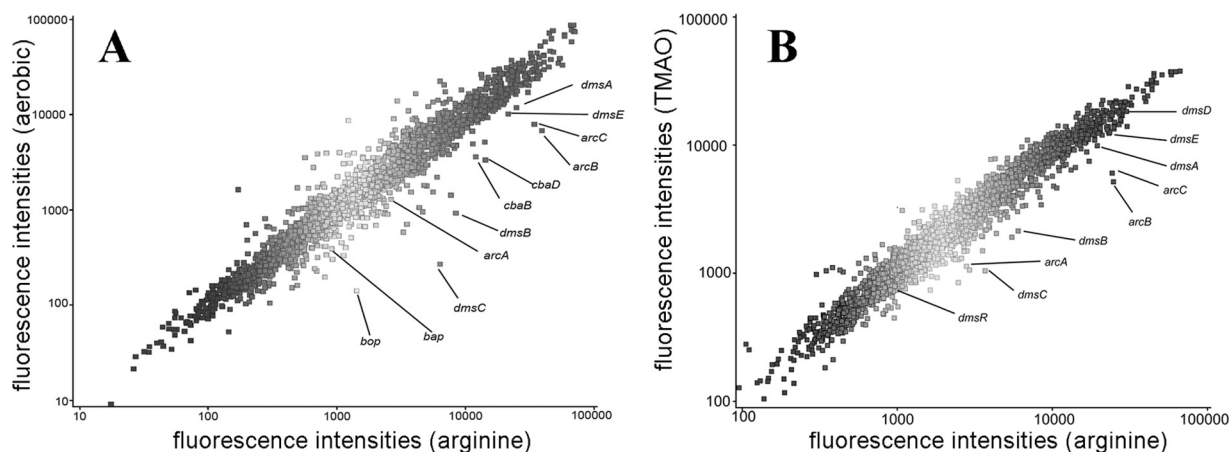


FIG 1 Genome-wide transcriptomic data comparing *Halobacterium* sp. NRC-1 grown (A) aerobically (y axis) versus anaerobically with arginine (x axis) or (B) anaerobically with TMAO (y axis) versus arginine (x axis). DNA microarray data (fluorescence units) were generated using cDNA probes fluorescence labeled with Cy3 or Cy5 and analyzed employing Agilent's GeneSpring program (see Materials and Methods). Metabolic genes with high transcript levels during arginine fermentation are labeled with gene names (see the text).

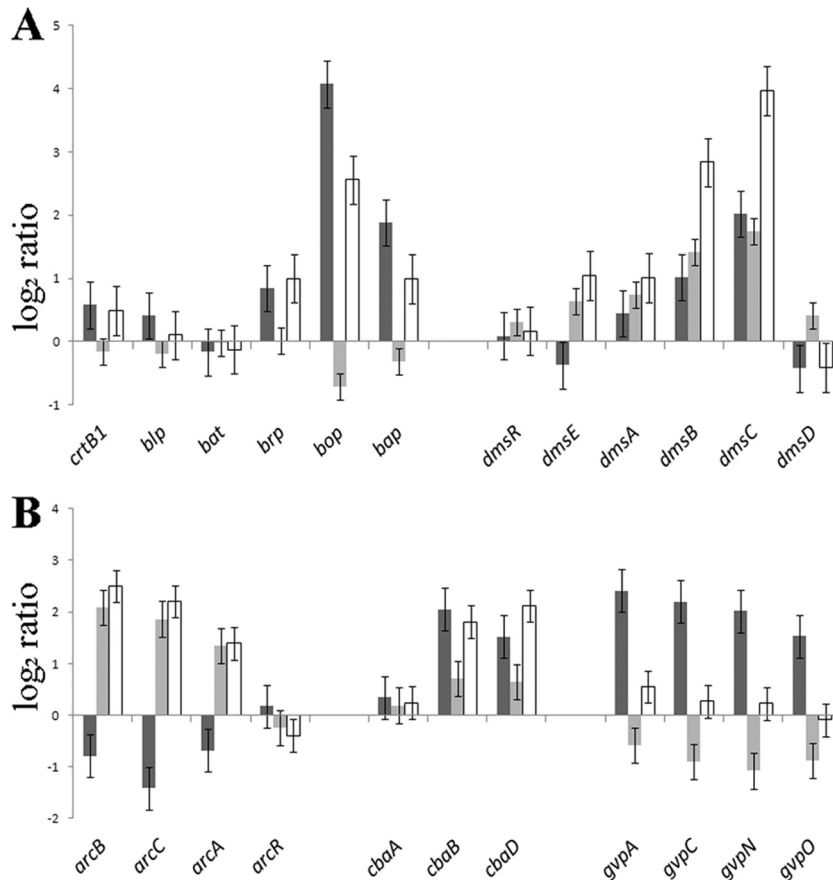


FIG 2 Gene expression changes in *Halobacterium* sp. NRC-1 grown under different conditions. Log₂ ratios (*y* axis) in gene expression are plotted for *bop* gene cluster and *dms* genes (A) and *arc* genes, *cba* genes, and *gvp* genes (B) (with the genes indicated below). Dark gray bars, anaerobic growth with TMAO versus aerobic growth; light gray bars, anaerobic growth with arginine versus anaerobic growth with TMAO; white bars, anaerobic growth with arginine versus aerobic growth. Error bars indicate standard errors.

compared to anaerobic respiration with TMAO. Compared to the differential expression of arginine fermentation versus aerobic growth, the degree of induction was nearly the same for *arc* genes but significantly less for *dms* genes. Like the *dms* genes, the *cba* genes also showed significantly greater induction with arginine than TMAO. In contrast, the *bop* gene, encoding the bacterio-opsin involved in phototrophic growth, and the transcriptionally linked *bap* gene, of unknown function, were downregulated under TMAO compared to their regulation under more reducing conditions in arginine. The other genes in the *bop* gene cluster, *crtB1*, *blp*, *brp*, and *bat*, were essentially unchanged in arginine versus TMAO. Like the *bop* and *bat* genes, the gas vesicle genes *gvpACNO* were downregulated in arginine compared to their regulation in TMAO. These results are consistent with previous findings comparing transcription during anaerobic respiration with TMAO to aerobic growth (Fig. 2, dark gray bars), showing coordinate induction of both purple membrane and gas vesicle synthesis under TMAO-reducing conditions. However, the finding of lower up-regulation of the *dms* operon during TMAO respiration than with arginine fermentation was unexpected (Fig. 2). These results are consistent with a gradient of transcriptional effects for metabolic genes. Under increasingly reducing conditions, *Halobacterium* sp. NRC-1 progressively induces the *gvp*, *bop*, *cba*, *dms*, and *arc* genes.

Regulatory mutants. In order to better understand the regu-

latory networks responding to oxygen limitation in *Halobacterium* sp. NRC-1, we targeted two positive regulatory genes of *Halobacterium* sp. NRC-1 for deletions, *bat* and *dmsR*, using the *ura3*-based deletion method (2). For the *bat* deletion mutant, we observed a slight difference in colony color for the mutant strain (pink) compared to that for the parent (more purple) on agar plates (Fig. 3A). Upon membrane extraction, purple membrane was detectable in the parent strain but was not detectable in the *bat* deletion mutant, while there was no observed difference in the red membrane fraction (Fig. 3B). DNA microarray analysis comparing the *bat* deletion mutant to the parent strain showed that expression of members of the *bat* gene cluster, including *crtB1*, *blp*, *brp*, *bop*, and *bap*, was also significantly lower in the mutant (Fig. 4, gray bars). These results are consistent with the purple membrane-deficient phenotype of the *bat* deletion mutant and confirmed the function of the *bat* gene in transcriptional activation of most of the genes near *bop*.

Previously, we had constructed a *dmsR* deletion strain and compared its anaerobic growth in the presence or absence of TMAO (Fig. 3C) (23). The results showed that while the wild-type *dmsR* strain can grow in medium with TMAO (Fig. 3C, blue curve) but not in the absence of TMAO (yellow curve), the Δ *dmsR* strain is unable to grow with TMAO (pink curve). We used DNA microarrays to compare the *dmsR* deletion strain to the parent

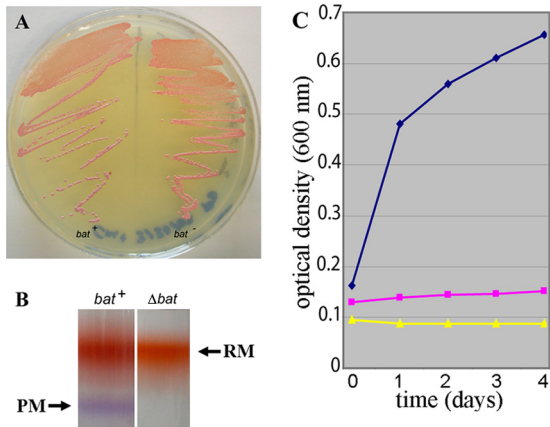


FIG 3 Phenotypes of regulatory mutants. (A) *Halobacterium* sp. NRC-1 *bat* wild-type (*bat*⁺) and *bat* deletion mutant (Δ *bat*) strains are shown on an agar plate. A subtle phenotypic difference is visible, with the wild-type *bat* strain appearing purple (left) and the *bat* mutant strain (right) appearing pink. (B) *Halobacterium* sp. NRC-1 wild-type *bat* (left) and *bat* mutant (right) red membrane (RM) and purple membrane (PM) fractionated by sucrose gradient. (C) Growth of *Halobacterium* sp. NRC-1 wild-type *dmsR* and Δ *dmsR* strains in anaerobic liquid cultures shown by plotting optical density (at 600 nm) versus time (in days). Shown are growth curves (average of 3 cultures) for the wild-type *dmsR* strain in medium with TMAO (blue) or without TMAO (yellow) and the Δ *dmsR* strain with TMAO (pink).

strain grown aerobically and found that genes in the *dmsR*-regulated operon, *dmsEABCD*, were significantly reduced in the *dmsR* deletion strain compared to the parental strain (Fig. 4, white bars). These results confirmed that the *dmsR* gene is a positive regulator of transcription of the *dms* operon.

Bioinformatic analysis of regulators. In order to discover other regulatory genes which may play a role in the response of *Halobacterium* sp. NRC-1 to oxygen-limiting conditions, we bioinformatically screened the *Halobacterium* sp. NRC-1 genome for genes encoding protein domains similar to those in the characterized regulatory genes *bat* (COG2202, COG2203, and COG3413) and *dmsR* (COG3413) (Fig. 5) (7, 8). Interestingly, we observed 10 predicted genes coding COG3413 HTH domains, one of which (*boa1*, a bacterio-opsin activator-like gene) also contained both COG2202 (LOV) and COG2203 (GAF) domains, like *bat*. In total, 12 LOV domain-containing genes were found, of which 5 contained GAF domains representing all of the members of the COG2203 GAF family. Several members of the COG2202 LOV family also contained signaling or sensor domains, including 8 out of 12 predicted histidine kinase domains (COG0642) in the *Halobacterium* sp. NRC-1 genome, 2 out of 6 with CheY receiver domains (COG0784), and 1 out of 17 with Tar methyl-accepting domains (COG0840). The *arcR* gene, encoding the regulator for the putative *arc* operon on pNRC200, was found to be unique, with a bacterium-type HTH domain (COG1414) in the N-terminal region (22). These bioinformatic results indicate that in addition to *bat* and *dmsR*, additional predicted regulatory genes likely participate in the anaerobic and microaerobic sensing and regulatory network for *Halobacterium* sp. NRC-1 (Fig. 5).

DISCUSSION

We have examined the transcriptional responses of the model halophilic archaeon *Halobacterium* sp. NRC-1 under different growth conditions comparing anaerobic growth with arginine fer-

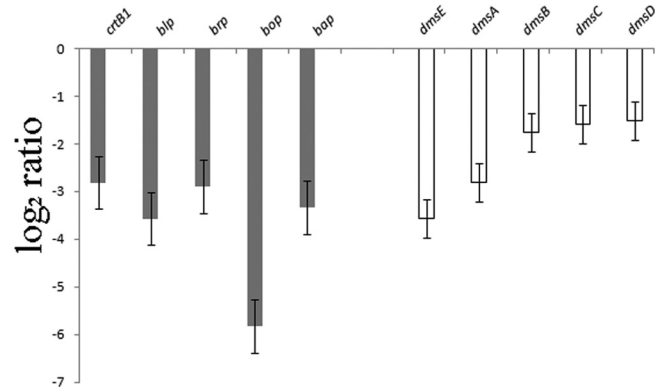


FIG 4 Gene expression in *Halobacterium* sp. NRC-1 Δ *bat* and Δ *dmsR* regulatory mutants. Log₂ ratios (y axis) between the Δ *bat* mutant (gray bars) or Δ *dmsR* mutant (white bars) and NRC-1 are shown for genes of the *bop* gene cluster and the *dms* operon (genes are indicated above). Error bars indicate standard errors.

mentation to either TMAO respiration or aerobic growth in rich medium. This work expands upon our previous study examining the transcriptional response of the same organism under TMAO-respiring conditions versus aerobic growth (23). In our studies, we also constructed in-frame deletions of two regulatory genes, *bat* and *dmsR*, which are linked to genes involved in the cellular response to anaerobic conditions, *bop* and *dms*, and determined the resulting phenotypic and transcriptional changes. Together, our work now provides a comprehensive genome-wide view of the response of a model archaeon to a common perturbation in environmental growth conditions, with transcriptional effects on genes encoding gas vesicles, purple membrane, an alternate cytochrome oxidase, DMSO/TMAO oxidoreductase, and ADI pathway detected.

A gradient of changes in transcript levels was observed in the order *bop*, *gvp*, *cba*, *dms*, and *arc*, genes which are involved in generation of metabolic energy under hypoxic and anoxic conditions, including fermentation (arginine) and anaerobic respiration (TMAO) (Fig. 2). The *arc* genes were most highly induced during arginine fermentation. Apparently, the determining factor driving the expression of the *arc* genes is a high concentration of arginine, since anaerobiosis alone does not result in differential expression of those genes (23). Surprisingly, the *dms* genes were most induced during growth via arginine fermentation rather than TMAO respiration. Assuming that all components of the electron transport chain are still present in the cell during arginine fermentation (see below), this regulatory scheme is consistent with the hypothesis that the redox state of cells governs expression of the *dms* genes in *Halobacterium* sp. NRC-1. The *cba* *ba*₃-type cytochrome oxidase genes were similarly induced during arginine fermentation and TMAO respiration in comparison to aerobic growth. The *bop* gene was also induced under anaerobic conditions, but not as dramatically in arginine as in TMAO. The *gvp* genes displayed a pattern similar to that for the *bop* gene (less induction with arginine and more with TMAO). These findings showed that cells in a partially reducing state (TMAO) induce the *bop*, *gvp*, *cba*, and *dms* genes in a gradient from more strongly to more weakly, with full induction of *arc* genes occurring in the presence of arginine.

Our whole-genome transcriptomic analysis also provided a

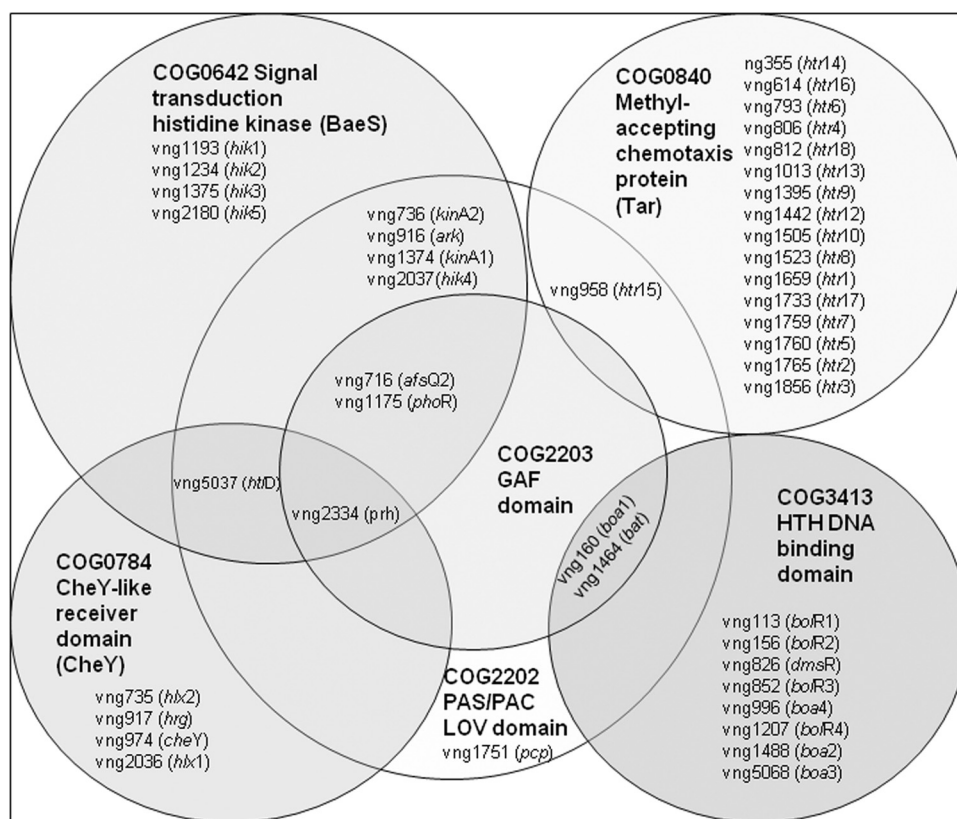


FIG 5 Venn diagram showing conserved protein family relationships for regulatory and signaling proteins in *Halobacterium* sp. NRC-1. COG designations are in bold for each family, and individual members of the family in *Halobacterium* sp. NRC-1 are indicated by vng numbers and gene names (in parentheses) (see reference 25).

broader picture of metabolic gene expression patterns under different growth conditions. Interestingly, the chromosomal cytochrome *c* oxidase genes (*cox*) were largely unchanged under the various growth conditions, in contrast to the cytochrome *ba*₃ oxidase (*cba*) and, to a lesser extent, the cytochrome *d* oxidase (*cyd*), coded on the inverted repeats of pNRC100 and pNRC200, which were induced during TMAO respiration. Among the other metabolic energy systems, electron transport chain components were largely unchanged. However, the ATP synthase genes were induced with oxygen when the growth rate was higher, as were nearly all of the genes of the tricarboxylic acid cycle. Similarly, the cobalamin biosynthesis genes were upregulated at the higher growth rate with oxygen, a likely reflection of the metabolic cost of cofactor synthesis (34). Not surprisingly, both superoxide dismutase (*sod*) genes involved in oxidative repair of DNA damage were upregulated as well in the presence of oxygen (6). Interestingly, an increase in the abundance of buoyant gas vesicle gene transcripts (*gvpACNO*) under oxygen-limiting conditions may enable flotation of cells to oxic zones in the water column, with the observed regulatory scheme for cytochrome *ba*₃ oxidase allowing scavenging of molecular oxygen whenever it becomes available (19, 23, 36).

Genetic and transcriptomic analyses of *bat* and *dmsR* confirmed and extended our understanding of the role of these regulatory genes in the anaerobic/redox response. The results clearly showed that *bat* and *dmsR* are each specific regulators of the nearby *bop* regulon and *dms* operon genes (Fig. 4). Transcrip-

tom results for the *dmsR* deletion mutant are consistent with previous findings and indicate the likely role in activation of the *dmsEABCD* operon (23). In contrast, the findings for the specific effect of the *bat* deletion on purple membrane genes are distinct from those for the spontaneous *bat* insertion mutation used in earlier studies, in which the mutants were pleiotropic and which resulted in the loss of both purple membrane and red bacterioruberin pigments. Previously, spontaneous *bat* insertion mutations resulted in a colorless or white phenotype, in contrast to the more restricted purple membrane-deficient pink phenotype for mutants with the constructed in-frame *bat* deletion (3, 35). A recent report also indicated that deletion of the *bop* gene significantly reduces bacterioruberin production (17). This disparity likely resulted from a strain-specific difference, such as the *bat* frameshift mutation reported in *Halobacterium* sp. strain S9 (1), in contrast to the wild-type background for strain NRC-1 in the present study. Expression of the retinal biosynthetic genes in the *bop* gene cluster (*crtB1* and *brp*) was also strongly downregulated in the *bat* deletion strain, but expression changes in these genes due to growth conditions were much less pronounced. A recently described gene located downstream from *bop*, named *bap*, coding a putative membrane protein (29), was also found to be downregulated in the strain with a *bat* gene deletion.

While the *bop*, *dms*, and *arc* structural genes were highly responsive to different growth conditions, expression of the nearby regulatory genes *bat*, *dmsR*, and *arcR* (the putative *arc* operon regulator) remained essentially unchanged. These findings show

that expression of transcriptional regulators does not need to be altered under different growth conditions and that their ability to affect gene expression likely occurs via changes in their protein structure and/or function. The LOV and GAF domains, which are predicted to sense redox and light, respectively, are presumably involved in Bat function (1, 17). For DmsR, where no LOV or GAF domains were identified, the N-terminal cysteine-rich region may play a role in redox sensing (23). For ArcR, which is a member of the IclR bacterium-type regulator family (COG1414) found in bacterial glycerol and acetate operons, no sensing domain has been identified (22).

Interestingly, the Bat and DmsR COG3413 HTH motif is primarily found in archaeon-type DNA binding proteins and may be quite prevalent, with 10 members in *Halobacterium* sp. NRC-1. Proteins in the COG3413 family are also found in *Sulfolobus* and *Thermoplasma* spp. (7, 8), which, like haloarchaea, are facultative anaerobes that can grow aerobically using organic compounds. These observations suggest that the COG3413 family may be involved in oxygen/redox regulation in other clades of facultative anaerobic *Archaea*. However, the COG3413 family does not appear to be exclusively used since other DNA binding domain proteins, like the N-terminal HTH-type domain in ArcR, are also involved in regulation of anaerobically induced genes.

The transcriptomic and genetic analyses in our studies have provided a detailed view of physiological changes in a model *Haloarchaeon* in response to environmental changes in oxygen and redox conditions. Together with studies of salinity, desiccation, radiation, and other environmental factors, we have gained a deeper understanding of how an extremophilic microorganism in the archaeal domain of life can be successful in a highly challenging and dynamic environment. Further detailed studies aimed at deciphering the regulatory networks operating in *Halobacterium* sp. NRC-1 are clearly necessary for a comprehensive understanding of the microbial physiology of this model organism.

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