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DNA microarray analysis of anaerobic *Methanosarcina barkeri* reveals responses to heat shock and air exposure

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Abstract *Methanosarcina barkeri* is a methanogenic archaeon that can digest cellulose and other polysaccharides to produce methane. It can only grow under strictly anoxic conditions, but which can survive air exposure. To obtain further knowledge of cellular changes occurring in *M. barkeri* in response to air exposure and other environmental stresses, we constructed the first oligonucleotide microarray for *M. barkeri* and used it to investigate the global transcriptomic responses of *M. barkeri* to air exposure and heat shock at 45°C for 1 h. The results showed that various house-keeping genes, such as genes involved in DNA replication recombination and repair, energy production and conversion, and protein turnover were regulated by environmental stimuli. In response to air exposure, up-regulation of a large number of transposase encoding genes was observed. However, no differential expression of genes encoding superoxide dismutase, catalase, non-specific peroxidases or thioredoxin reductase was observed in response to air exposure, implying that no significant level of reactive oxygen species has been formed under air exposure. In response to heat shock, both Hsp70 (DnaK-DnaJ-GrpE chaperone system) the Hsp60 (GroEL) systems were up-regulated, suggesting that they may play an important role in protein biogenesis in *M. barkeri* during heat stress.

Keywords Microarray · Stress · *Methanosarcina barkeri*

Supplementary table including all the genes in the *M. barkeri* genome and their response to heat shock and air exposure will be provided upon request.

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Introduction

Methanogenic archaea have an unusual type of metabolism because they use H_2 + CO_2, formate, methylated C1 compounds, or acetate as energy and carbon sources for growth. One of the important applications of this group of microorganisms is that they can digest cellulose and other polysaccharides for methane production. In a unique energy-generating process, methanogens produce methane as the major end product of their metabolism. These organisms have also received much attention because they are essential for both the recycling of carbon compounds and the maintenance of the global carbon flux [19, 33]. Furthermore, methane is an important greenhouse gas that directly contributes to climate changes and global warming. Previous studies have shown that *Methanosarcina barkeri* can only reproduce under strictly anoxic conditions, but that it can survive periods of oxidative stress. The death rate of *M. barkeri* in air was low [38], and *Methanosarcina* species in oxic paddy field soils rapidly began to produce methane and to grow again as soon as anoxic conditions were re-established [8]. However, little is known about the molecular basis underlying the response of this microorganism to various environmental perturbations.

Under aerobic conditions, generation of highly toxic and reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radicals, leads to oxidative stress with deleterious effects on the growth of many organisms [2, 28]. The main detoxification system for reactive oxygen species in aerobic and anaerobic bacteria involves superoxide dismutase (Sod) to convert superoxide radicals to peroxides followed by the action of catalases (KatA, KatG) and nonspecific peroxidases (for example, AhpC) [28] to further convert these peroxides to water and oxygen. Although a monofunctional catalase and an iron superoxide dismutase were previously purified from the archaeal *M. barkeri* [1, 31], the

metabolic and transcriptional adaptation of *M. barkeri* to oxygen stress remains to be elucidated.

The genome of *M. barkeri* is being sequenced through the DOE Joint Genome Institute and, thus far, the draft genome, with a total length of 4.87 Mb, consists of 3,830 candidate protein-coding genes (http://www.img.jgi.doe.gov/pub/main.cgi). Benefiting from these sequencing efforts, the first whole genome oligonucleotide microarray was constructed and utilized to characterize the global transcriptomic changes occurring in *M. barkeri* when subjected to air exposure and heat shock, two most common stresses in natural environments. The objective of this investigation was to use microarray analysis to determine changes in abundance of transcripts in response to these two environmental stresses.

Materials and methods

Strains, media and culture conditions

Methanosarcina barkeri DSM 805 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSM) and grown under strictly anaerobic conditions as described previously [4]. Cells were grown at 30°C in minimal medium, consisting of standard *Methanosarcina* medium (DSMZ 120) without casitone. Growth experiments were performed in 140 ml serum bottles containing 70 ml of medium using 125 mM methanol as the carbon source. After inoculation (10%) from a preculture grown under the same medium, cultures were incubated at 30°C for 2–3 days until reaching mid-log phase, corresponding to an optical density of approximately 0.5 at 590 nm, as determined in a Shimadzu BioSpec 1601 Analyzer (Kyoto, Japan). For air exposure treatments, the cultures were exposed to continuous bubbling with air for 1 h. For the heat shock treatments, the cultures were incubated in a 45°C water bath for 1 h. The cell growth was checked before and after the stress treatments. Tubes containing approximately 100 µl of packed cells were then frozen in liquid nitrogen and stored at −80°C until extraction. Each sample used for RNA profiling by microarray analysis was a pool of three individual biological replicates. Two samples were collected for each condition. All operation involved sample collection was performed under anaerobic conditions.

Generation of the *M. barkeri* array

Microarrays were designed by NimbleGen System Inc. (Madison, WI, USA) using its Maskless Array Synthesizer technology [25]. The array was designed and manufactured as described before [25]. For each ORF, 18 unique 24-mer oligonucleotides distributed throughout the ORF were printed onto glass microscope slides.

RNA isolation and microarray analysis

Total RNA was isolated from frozen *M. barkeri* cell pellet (150–200 µl) using a protocol developed in our laboratory. Briefly, the cell pellets were added to a mortar containing liquid nitrogen and ground to a fine powder. One milliliter of Trizol reagent (Invitrogen, Carlsbad, CA, USA) was immediately added to the powder in the pestle and allowed to thaw in the mortar. The resulting slurry was transferred to a 2 ml O-ring tube containing 100 µl each of 0.5 and 0.1 mm glass/zirconia beads and homogenized for 6 min (2×3 min with 5 min rest between) in a Mini-Bead-Beater-8 Cell Disruptor (Biospec Products) at maximum speed. The tubes were then incubated at room temperature for 5 min before adding 200 µl of chloroform, vortexing for 15 s and centrifuging at 12,000×*g* for 15 min at room temperature. The aqueous layer (~600 µl) was transferred to a tube containing 600 µl of 2-propanol, mixed and incubated at room temperature for 15 min before centrifuging at 12,000×*g* for 15 min at 4°C. The pellet was washed with 70% ethanol, air dried and re-suspended in 50–100 µl of RNase-free water by heating to 60°C for 10 min. The concentration and purity of the RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the integrity was verified by gel electrophoresis. The quality of total RNA obtained was re-evaluated immediately prior to the biotin labeling reaction using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

RNA biotin-labeling and hybridization and washing of the microarray

The total RNA (10 µg) of *M. barkeri* was labeled with biotinylated ddATP. The labeling, array hybridization, array washing and scanning were performed as described before [25].

Microarray data normalization and gene expression analysis

Prior to data extraction, images were rotated and doubled in sizes (without interpolation) using ImageJ software (http://www.rsb.info.nih.gov/ij/). Features were extracted using GenePix 3.0 software (Axon Instruments, Inc.), using a fixed feature size. The local background correction from the GenePix software was not applied to raw signal intensities. The data was normalized using tools available through the Bioconductor project (http://www.bioconductor.org) as previously described [25]. The gene calls were based on the Bioconductor implementation of the MAS 5 algorithms [25]. For each treatment condition, two replicate RNA labeling reactions and hybridizations were performed (using RNA from a pool of three biological replicates). Genes were considered as having a changed expression if

they had an mRNA concentration that changed 2.0-fold or more when compared to untreated samples. In addition, confidence level (*P*-value) of expression changes was calculated and only those with confidence level greater than 90% were considered to be significant.

Results and discussion

Overview of gene expression under stress conditions

Methanosarcina barkeri cells used in gene expression profiling were grown in a methanol based medium and collected from mid-exponential phase cultures corresponding to an OD_{590} of approximately 0.5. In this study, the cells were then subjected to air exposure by continuous bubbling with air for 1 hour or to heat shock treatment by incubating in a 45°C water bath for 1 h, in order to make the stress condition comparable to several previous studies on *Methansarcina* strains, which showed that mRNA levels of heat shock protein DnaK reached a high level already after a heat shock at 45°C for 30 min, with only slight increases at 50, 55, and 60°C in *M. mazei* [5], and in thermophile *M. thermophila* TM-1, the detection of the *dnaK* gene required at least 30 min exposure to at least 60°C and the highest levels of *dnaK* mRNAs were induced by a 1-h heat shock [18].

Pair-wise comparison of gene expression patterns in control and the stress-treated samples showed that significant transcriptional changes occurred in response to these stressors. Using a cutoff criterion of 2.0 fold change and 90% confidence when compared to untreated samples, a total of 544 genes were identified as responsive to air exposure and 168 genes were responsive to heat shock treatment. Responsive genes involved in almost all aspects of *M. barkeri* metabolism were found (Table 1).

Genes responsive to heat shock

A total of 168 genes were regulated by heat shock in *M. barkeri*, including 79 down-regulated genes and 89 up-regulated genes. Functional analysis showed that genes involved in amino acid, coenzyme and energy metabolism were down-regulated (Table 1). For example, four subunits of genes encoding the archaeal A_1A_0 ATPase were don-regulated by heat shock. The top category induced by heat shock in *M. barkeri* was genes playing critical roles in physiological protein biogenesis (Supplementary table).

It was established only recently in *M. mazei* that archaea have a chaperonin (Hsp60) system like that of the other prokaryotes [6, 15, 21] In bacteria, it has been suggested that the Hsp70 (DnaK-DnaJ-GrpE chaperone system) and Hsp60 (GroEL) systems coexisted and seemingly evolved together to interact with each other. The Hsp70 system acts early in protein biogenesis to avoid aggregation of nascent polypeptides during translation or immediately thereafter. Subsequently, the polypeptide reaches the GroEL/S system for final folding [21, 23]. While in *M. mazei*, it has been suggested that each type of chaperonin systems may assist in the folding of a subset of cytosolic proteins, with GroEL/GroES likely being essential for the folding of some of the proteins of bacterial origin [15]. Microarray analysis showed that DnaJ, DnaK and GrpE genes of Hsp70 chaperone system were induced ~2.2 fold by heat shock (Supplementary table). In addition, analysis of the draft genome sequence of *M. barkeri* identified four genes with high identity to *groEL* genes (with *E*-value < 1e-125), and transcriptomic analysis showed that two of the genes, MbraA_1201 and MbraA_1543 were induced by heat shock, suggesting that a mechanism based on Hsp60 played important roles in stress response to heat shock in archaeal *M. barkeri*. Just before our study, the first microarray analysis of heat shock response in *Archaea* was done just recently in hyperthermophilic archaeon *Archaeoglobus fulgidus* [29]. The analysis showed that expression of a total of 14% of the genes in the *A. fulgidus* genome was differentially regulated after 60 min of heat shock treatment. While there is no Hsp70 gene annotated in *A. fulgidus*, the genes encoding the HSP60s and the small heat shock protein (sHsp20) were heat induced [29].

In addition, several other genes involved in protein biogenesis, including another two genes encoding molecular chaperone (putative heat shock protein MbarA_0907 and MbarA_1806), a gene with similarity to ATPases of the AAA+ class (MbarA_0908), a gene similar to bacterial type ATP-dependent La protease (MbarA_2576) and two genes encoding peptidylprolyl isomerase (MbarA_1939 and MbarA_1941) were induced by heat shock (Supplementary table). The ATPases of the AAA+ class included enzymes containing a P-loop NTPase domain, and function as molecular chaperones, ATPase subunits of proteases, helicases or nucleic-acid-stimulated ATPases [14]. The bacterial type ATP-dependent La protease was heat-shock inducible in *E. coli* and prevented the accumulation of damaged cellular protein [11]. In *A. fulgidus*, while a La homolog without the ATP binding domain was not differentially expressed upon heat shock, a proteosome beta subunit (PsmB), a subtilisin-like peptidases (AF1652) and a heat shock protein X (AF0235) were induced [29]. Peptidylprolyl isomerase (PPIase) has been identified as mediators of protein folding and assembly in vivo. It catalyses the *cis–trans* isomerization of the proline imide bond in polypeptides, which is the rate-limiting step in protein folding, and consequently accelerate protein folding [17]. In archaeal *M. thermolithotrophicus* and *Thermococcus* sp. KS-1, MtFKBP17 and TcFKBP18 both with PPIase activity were chaperones involved in refolding unfolded proteins *in vitro* [10, 12]

Involvement of two-component signal transduction systems in regulation of stress responses has been reported in many organisms [22, 27, 35]. Transcriptomic analysis of heat shock response in *M. barkeri* showed that several genes involved in regulatory function were significantly

Table 1 Genes differentially expressed in response to heat shock and air exposure

Functional category of responsive genes	Heat shock				Air exposure			
	≥2.0 fold		≥3.0 fold		≥2.0 fold		≥3.0 fold	
	No.[a]	%[b]	No.	%	No.	%	No.	%
Amino acid transport and metabolism	11	4.60	3	1.26	47	19.67	11	4.60
Carbohydrate transport and metabolism					10	10.99	1	1.10
Cell division and chromosome partitioning	1	4.00	1	4.00	4	16.00		
Cell envelope biogenesis outer membrane	1	0.86			15	12.93	1	0.86
Coenzyme metabolism	12	7.10	4	2.37	23	13.61	4	2.37
Defense mechanisms					5	8.33	2	3.33
DNA replication recombination and repair	21	9.33	1	0.44	58	25.78	21	9.33
Energy production and conversion	15	6.25	2	0.83	31	12.92	7	2.92
Function unknown	63	3.83	11	0.78	131	9.30	37	2.63
General function prediction only	18	3.89	4	0.86	62	13.39	14	3.02
Inorganic ion transport and metabolism	8	4.52	3	1.69	21	11.86	8	4.52
Intracellular trafficking and secretion	2	6.25			7	21.88	2	6.25
Lipid metabolism					4	12.90	1	3.23
Nucleotide transport and metabolism	1	1.64			14	22.95	4	6.56
Posttranslational modification protein turnover chaperones	13	12.15	6	5.61	29	27.10		
Secondary metabolites biosynthesis transport and catabolism					2	7.69		
Signal transduction mechanisms	2	2.90	1	1.45	4	5.80		
Transcription	8	5.84	3	2.19	27	19.71	4	2.92
Translation ribosomal structure and biogenesis	1	0.57			50	28.74	7	4.02
Total	168		39		544		124	

[a]The number represents the actual number of responsive genes in that category
[b]Representing the percentage of responsive genes against the total number of genes in that category

up-regulated, which included MbarA_1535 encoding a putative sensor histidine kinase and three genes (MbarA1707, MbarA1709 and MbarA2575) encoding transcriptional regulators (Supplementary table). Among them, MbarA_2575 encoding a transcriptional regulator was located immediately upstream of MbarA_2576 encoding a bacterial type ATP-dependent Lon protease, while MbarA_0909 encoding a predicated transcriptional regulator was located immediately upstream of MbarA_0908 encoding an ATPases of the AAA + class and MbarA_0907 encoding a molecular chaperone (small heat shock protein), suggesting they may be directly involved in regulation of heat shock response.

Upon heat shock, 16 genes encoding putative transposases were up-regulated (Supplementary table). Transposases that catalyze translocation various mobile DNA sequences, have been found up-regulated by heat-shock in many microorganisms [26, 34].

Genes exclusively responsive to air exposure

A total of 544 genes were differentially regulated by air exposure in *M. barkeri*, which included 90 genes also responsive to heat shock stress. Among 454 genes exclusively responsive to air exposure, 401 genes were down-regulated and 53 genes up-regulated. Functional analysis showed that the most down-regulated genes were those involved in housekeeping function (Supplementary table). In term of the number of genes affected, the top category down-regulated by oxidative stress was genes involved in translation, ribosomal structure and biogenesis function, which included some of the ribosomal protein genes, translation initiation factors (IF1 and IF2), and translation elongation factors, and several aminoacyl-tRNA synthetases, suggesting that when confronted with oxidative stress, the overall protein biosynthesis was slowed down (Supplementary table). Other major categories down-regulated by air exposure included genes involved in amino acid transport and metabolism, energy production and conversion, coenzyme metabolism and transcriptional regulation and signal transduction (Supplementary table). A similar distribution pattern was found for the top 40 genes whose expressions were down-regulated only by oxidative stress (Table 2).

Several mechanisms of oxygen resistance and reduction have been described in bacteria. In addition to the main detoxification systems of superoxide dismutase (Sod), catalases (KatA, KatG) and nonspecific peroxidases (for example, AhpC) [28], mechanisms involving rubredoxin oxidoreductase (Rbo), rubrerythrin (Rbr) and thioredoxin reductase (TrxB) have also been suggested to be alternative methods for protecting against oxidative stress in anaerobic *Desulfovibrio* species [9, 20, 37]. However, transcriptomic analysis showed none of these genes was differentially regulated in *M. barkeri* when subjected to air exposure [28, 30, 36]. To determine if these genes were constitutively expressed in *M. barkeri*, the absolute expression data (signal intensity) of these transcripts was examined. The result showed that one gene encoding a putative superoxide dismutase in *M. barkeri* genome, MbarA_3589 (SodA), and one gene encoding a putative peroxiredoxin (AhpC),

Table 2 Top 40 genes whose expressions were down-regulated only by air exposure

Gene ID	Ratio by air exposure	*P* value	Description
Amino acid transport and metabolism			
MbarA_0159	−3.42	0.0354	Nitrogen regulatory protein P-II
MbarA_0469	−3.59	0.0867	Nickel ABC transporter, permease protein
MbarA_0587	−5.80	0.0944	Amino-acid acetyltransferase
MbarA_0666	−3.50	0.0639	Glutamine synthetase
MbarA_0923	−3.99	0.0123	Shikimate 5-dehydrogenase
MbarA_1067	−3.64	0.0310	Hydantoinase
MbarA_2177	−5.03	0.0369	Ornithine decarboxylase
MbarA_2599	−3.49	0.0923	Putrescine-ornithine antiporter
MbarA_3623	−5.16	0.0191	Tryptophan synthase, subunit alpha
MbarA_3624	−5.73	0.0146	Anthranilate phosphoribosyltransferase
Coenzyme metabolism			
MbarA_0211	−3.50	0.0256	Coenzyme F390 synthetase
MbarA_1493	−3.55	0.0298	Cobalamin biosynthesis protein CbiD
Cell envelope biogenesis outer membrane			
MbarA_1137	−3.21	0.0618	Aspartate aminotransferase
Defense mechanisms			
MbarA_3207	−3.67	0.0279	Putative transmembrane efflux protein
Energy production and conversion			
MbarA_0881	−3.77	0.0953	Coenzyme F420 hydrogenase, beta subunit
MbarA_1419	–4.31	0.0715	Isopentenyl-diphosphate delta-isomerase
MbarA_2418	–3.33	0.0670	Fumarate hydratase, alpha subunit
MbarA_2420	–3.95	0.0629	Phosphorylase
General function prediction only			
MbarA_0137	–3.56	0.0494	Succinoglycan biosynthesis regulator
MbarA_0574	–3.70	0.0334	GTP-binding protein
MbarA_0885	–3.51	0.0389	Carbon–nitrogen hydrolase
MbarA_1420	–3.33	0.0116	Amino acid kinase
MbarA_1984	–3.66	0.0621	Haloacid dehalogenase-like hydrolase
MbarA_3358	–4.62	0.0617	Putative ABC-2 type transport system permease protein
MbarA_3420	−3.38	0.0368	MoaA/NifB/PqqE family protein
Inorganic ion transport and metabolism			
MbarA_0158	−5.35	0.0196	Ammonium transporter
MbarA_0589	−3.50	0.0462	Ferrous iron transport protein B
MbarA_1176	−3.36	0.0160	ABC transporter, solute-binding protein
MbarA_2563	−3.29	0.0384	Cobalt transport protein
MbarA_3372	−5.00	0.0615	Cell surface protein
Intracellular trafficking and secretion			
MbarA_0087	−3.62	0.0082	Protein translocase subunit SecY
Nucleotide transport and metabolism			
MbarA_1178	−3.38	0.0098	GMP synthase [glutamine-hydrolyzing]
MbarA_3515	−3.36	0.0413	Orotate phosphoribosyltransferase
Transcription			
MbarA_3692	−3.55	0.0862	DNA-directed RNA polymerase, subunit A
Translation ribosomal structure and biogenesis			
MbarA_0883	−3.76	0.0808	Glutamyl-tRNA(Gln) amidotransferase, subunit A
MbarA_1205	−3.41	0.0174	Translin family protein
MbarA_1374	−3.29	0.0234	Tryptophanyl-tRNA synthetase
MbarA_2366	−3.65	0.0134	Translation initiation factor 1A
MbarA_3159	−3.40	0.0729	FtsJ-like methyltransferase
MbarA_3690	−3.22	0.0197	Ribosomal protein L30e

The list does not include functionally unknown genes in the top 40

MbarA_0479, were highly expressed in both control and air exposure-treated samples with an average intensity of 1,631 and 2,303 arbitrary units, respectively. In contrast, expression of the genes encoding a putative catalase and a thioredoxin reductase was low under all conditions tested (Supplementary table). The result suggested that maybe only low concentration of ROS was formed after exposed to air for 1 h, and thus no gene involved in combating ROS needs to be induced for the survival of *M. barkeri*.

A gene encoding a Na^+-driven multidrug efflux pump, MbarA_3744 was up-regulated by 3.45 fold in response to air exposure in *M. barkeri* (Supplementary table). Previously, the cation efflux pump CzcD was hypothesized to be involved in long-term survival of the obligate anaerobe *Bacteroides fragilis* in the presence of

oxygen [32], suggesting that MbarA_3744 may also be involved in resistance against oxidative stress. Interestingly, the CzcD protein was also implicated in resistance against stress caused by metal ions [24].

Another noteworthy feature occurring in response to air exposure was the significant up-regulation of genes encoding transposases. Among the 79 genes (including 25 unknown genes) up-regulated by oxidative stress, 45 genes encoded various putative transposases (Supplementary table). It has been shown that different stresses, such as carbon starvation [13, 16], temperature effects [26], and UV light [7] can enhance transposition of bacterial mobile elements [3]. Recent evidence in *Pseudomonas putida* showed that transcription from the Tn*4652* transposase promoter is controlled by the stationary-phase-specific sigma factor σ^S, suggesting that activation of transposition under stressful conditions could be an inducible process [13]. To our knowledge, this is the first example of induction of mobile element transposase gene expression by air exposure or oxidative stress. The large number of transposase genes induced in *M. barkeri* in response to air exposure suggests that transposon movement may similarly be induced by this stress. Transposons have been demonstrated to be involved in mutational divergence in long-term cultures of *E. coli* and were hypothesized to be a factor in bacterial evolution [39]. Although further experiments would obviously be required to determine if transposon induced mutations are also occurring in *M. barkeri* in response to air exposure, the large number of transposon genes up-regulated during this response makes it tempting to speculate that transposon movement may constitute an important aspect of stress inducible or adaptive mutation in this organism.

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