

Microarray Analysis of Transcriptomic Response of *Escherichia coli* to Nonthermal Plasma-Treated PBS Solution

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Abstract

We developed a technique of generating nonthermal atmospheric plasma-activated solution that had broad-spectrum antibacterial properties. Plasma-activated phosphate-buffered saline (PBS) causes rapid inactivation of bacteria following generation of oxidative stress. However, dose optimization requires understanding of cellular mechanisms. The objective of this study was to explore genome-wise response to develop gene expression profile of *Escherichia coli* using DNA microarray following exposure to plasma-activated PBS solution. Upon exposure to plasma-treated PBS solution, *E. coli* cells had differentially expressed genes involved in oxidative stress, and cell envelope and membrane associated porin and transporters. The genes involved in house-keeping and metabolism, energy generation, motility and virulence were conversely downregulated. This is the first report which demonstrates a severe oxidative stress induced in *E. coli* cells in response to an exposure to nonequilibrium nonthermal dielectric-barrier discharge plasma-activated PBS solution, and the genes that are responsive to reactive oxygen species appeared to play a role in cellular stress. Such studies are important to identify targets of inactivation, and to understand plasma-treated solution and bacterial cell interactions.

Keywords

Antibacterial Solution, Disinfection, *Escherichia coli*, Gene Expression, Indirect Plasma, Microarray, Nonthermal Plasma, Transcriptomics

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1. Introduction

Nonthermal nonequilibrium atmospheric plasma (plasma) is being investigated for disinfection and sterilization processes in biology and medicine. Since last decade, several publications appeared which stressed the advantages of this technique over traditional disinfection techniques [1] [2]. The direct plasma application technique is a little challenging to delicate surfaces and tissues, which are likely to be damaged by high energy electrons and UV photons generated during direct bombardment of plasma [3]-[6]. In addition, the distance between plasma generating probe and the surface being treated is critical, and determines the dose and efficacy of plasma.

Recently, we have demonstrated a technique of applying plasma indirectly wherein the plasma generating probe doesn't come in contact with such surface or the surface of skin or mucous membranes, and is highly portable and does not require gas or air cylinders and the associated assembly (unlike jet or afterglow plasma technique). We have developed plasma-treated solutions that retain strong antimicrobial property for up to two years of time period [7]. The antibiotic-like solutions are fast acting and require only about 180 seconds of plasma treatment for activation of antibacterial property, and less than 15 min of contact (holding) time is sufficient to inactivate bacterial pathogens [7]. A plasma-activated phosphate-buffered saline (PBS) solution is one of the strong oxidative biocidal agents we generated, which has powerful antibacterial and antibiofilm properties. Upon plasma exposure, certain oxidizing chemical species are produced in PBS solutions which are responsible for rapid inactivation of bacteria [7] [8]. Based on our preliminary observations, we anticipate that oxidative stress is responsible for rapid cell death, but the exact mechanism of bacterial inactivation is not known. We also anticipate that plasma-activated PBS solution may have multiple targets, primarily related to oxidative stress-mediated changes. A systematic genome-wise analysis is required to define such targets of inactivation.

Genomic analysis is one of the most favored approaches of study underlying genetic mechanisms of inactivation and regulatory response of genes that govern cell death. Cellular defenses have their thresholds and beyond this limit cells cannot survive stress conditions. *Escherichia coli* is one of the suitable model bacteria for this study whose whole genome sequence is available and metabolic pathways are well studied [3] [9]. DNA microarray is a widely used molecular technique which allows differentiating gene expression under various conditions, to monitor patterns of global gene expression, and thus helps in understanding cellular processes. In addition, microarrays are much more convenient to work with than DNA/RNA membrane blotting [10]. Aim of this study was to explore the gene expression profile responses of global genes in *E. coli* that occur upon exposure to plasma-activated antimicrobial PBS solution, to predict related transcriptomic changes.

2. Materials and Methods

2.1. Bacterial Culture and Condition

Escherichia coli (ATCC-29522) was used for this study. The culture was developed by inoculation of a single isolated colony from overnight grown trypticase soy agar plate into 10 ml of trypticase soy broth (TSB) and incubated at 37°C in a stationary incubator. On the following day, the culture was reinoculated and the growth monitored by taking optical density (OD₆₀₀) of the culture aliquots.

2.2. Chemical Solution, Plasma Setting, and Treatment Conditions

Phosphate-buffered saline solution (PBS; Sigma; 150 mmol/L sodium chloride and 150 mmol/L sodium phosphate, pH 7.2, at 25°C) was prepared using deionized water (MP Biomedicals Inc., Solon, OH). Solution was freshly prepared, 0.22 µ filter sterilized, aseptically handled; and aliquot either used fresh or stored at -20°C for subsequent experiments. Similarly, α -tocopherol (vitamin E; 200 mM), thiourea and catalase (all from Sigma-Aldrich, St. Louis, MO, USA) were prepared as stock solutions in untreated PBS buffer, filter sterilized, and used at predetermined concentrations (α -tocopherol, 200 mM; thiourea, 50 mM; catalase, 200 units). Nonthermal plasma generator was used in this study, and the in-house built electrode and fluid chamber system was reported earlier by our laboratory [7]. The parameters were set to 11 kV, 0.26 W/cm², 1000 Hz pulse frequency, 2 mm of gap between the surface of fluid and DBD electrode. One milliliter of chemical solution was treated for 15 seconds through 180 seconds or left untreated [zero (0) second], and used freshly.

2.3. Bacterial Inactivation Assays

Halfmilliliter (0.5 ml) of PBS solution treated with plasma for various duration of treatment was mixed with 0.5

ml of *E. coli* cell suspension (1 ml culture of 0.2 OD₆₀₀ was centrifuged at 4000 revolutions per minute (RPM), cell pellet was washed twice with sterile PBS, and resuspended in 0.5 ml of PBS), and held for 15 min of contact time. The cells were harvested by centrifugation, and resuspended in untreated PBS to proceed for XTT assay using XTT reagents (Molecular Probes) as described earlier [11] [12] to evaluate the treatment-time-dependent oxidative stress mediated antimicrobial efficacy of plasma-treated PBS and effect of ROS scavengers. A hydrogen peroxide (0.3%) reagent and plasma untreated PBS solution were used as positive and negative controls respectively. The assays were repeated three times to confirm findings.

2.4. Hydrogen Peroxide Detection Assay

The amount of hydrogen peroxide retained in each sample was measured using Hydrogen Peroxide Detection Kit (National Diagnostics, Atlanta, GA) following manufacturer's protocol. In brief, a working solution of 20 ml was prepared by combining two reagents supplied in the Kit, and the serial dilutions of standard hydrogen peroxide were tested to generate standard curve. In parallel, the undiluted or serially diluted plasma-activated PBS was prepared, and H₂O₂ detection assay performed in 96 wells plate in triplicate. The assay was repeated twice in triplicate and H₂O₂ concentrations determined as per manufacturer's protocol.

2.5. Microarray Assays

A predetermined (60 seconds) dose of plasma treatment was used to activate PBS solution. One ml exponential *E. coli* culture (0.2 of OD₆₀₀) was centrifuged at 4000 RPM to harvest cells and the cell pellet was washed twice with sterile PBS and resuspended in 500 µl of PBS. An equal amount (500 µl) of plasma-treated or untreated PBS solution or H₂O₂ solution was then added to this cell suspension and the reaction mix was held for 15 min (contact time), after which cells were suspended in RNA later reagent (Qiagen, Valencia, CA) to stabilize RNA, and then were pelleted again by centrifugation, and subjected to cell lysis using RNeasy mini kits (Qiagen) following the manufacturer's protocol. RNA was isolated from *E. coli* cells exposed to either plasma-treated or untreated fluid or H₂O₂ solution, and quantified on a NanoDrop spectrophotometer (Thermo Scientific), followed by RNA quality assessment on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). Amplification and labeling was performed using the Ovation Pico WTA-system V2 RNA amplification system (NuGen Technologies, Inc.). Briefly, 50 ng of total RNA was reverse transcribed using a chimeric cDNA/mRNA primer, and a second complementary cDNA strand was synthesized. Purified cDNA was then amplified with ribo-SPIA enzyme and SPIA DNA/RNA primers (NuGen). Amplified c-DNA was purified with Qiagen MinElute reaction cleanup kit. The concentration of Purified cDNA was measured using the NanoDrop. The cDNAs (2.5 µg) were fragmented and chemically labeled with biotin to generate biotinylated cDNA using FL-Ovation cDNA biotin module V2 (NuGen). Affymetrix Genechip® *E. coli* Genome 2.0 array system (Affymetrix, Santa Clara, CA) was used. The product was hybridized with fragmented and biotin-labeled target (2.5 µg) in 110 µl of hybridization cocktail. Target denaturation was performed at 99°C for 2 min and then 45°C for 5 min, followed by hybridization for 18 h. Arrays were then washed and stained using Gene chip Fluidic Station 450, and hybridization signals were amplified using antibody amplification with goat IgG and anti-streptavidin biotinylated antibody. A hydrogen peroxide (0.3%) reagent and plasma untreated PBS solution were used as positive and negative controls respectively.

2.6. Data Analysis

Gene-Chips were scanned on an Affymetrix Gene Chip Scanner 3000, using Command Console Software. Background correction and normalization were done using Iterative Plier 16 with Gene Spring V11.5 software (Agilent). A list of differentially expressed genes (in fold) was generated for the genes whose transcription is significantly influenced, and the list was loaded into Ingenuity Pathway Analysis (IPA) 5.0 software (<http://www.ingenuity.com>) to perform biological network and functional analyses. The transcript expression values of treated sample array (plasma-treated PBS) versus plasma untreated sample array were considered significant when the difference ratio was 1.2, and subsequently, we selected genes which were differentially expressed by >2 fold (against untreated samples). Experiments were repeated in triplicate, and a mean of fold expressions shown.

3. Results

3.1. Plasma-Activated PBS Doses, Bacterial Inactivation and Oxidative Stress

The plasma generating setup used in these studies is published earlier [3] [7] [11]. Earlier, we reported that inactivation of bacteria is plasma-treatment (plasma energy)-dependent [7], and therefore only the representative growth inhibition curves, and the effect of ROS scavengers in restoring cells from oxidative stress are provided here (Figure 1). During optimization, we determined that 1 ml of plasma-treated PBS solution for 60 seconds inactivate number of *E. coli* only by one quarter; hence we used predetermined 60 seconds treatment of PBS solution as sublethal dose for this microarray analysis study (details of the killing curves and colony count assay are published earlier and hence are not provided here). Figure 1 shows the representative graphs of plasma-treatment times-dependent *E. coli* growth inhibition, and the effect of anti-oxidants, α -tocopherol (vitamin E), catalase, and thiourea on scavenging ROS, leading to significant protection of cells from cellular inactivation ($*P \leq 0.05$) as compared to corresponding non-scavenged conditions of both, 1 minute and 2 minute plasma-treated PBS. The hydrogen peroxide assay revealed that the amount of H_2O_2 generated in solution at 60 seconds (sub-lethal dose) was 0.42 mM (± 0.03 mM) (an average of three readings).

3.2. Plasma-Activated PBS Solution and Expression Profile of Global Genes

By whole genome approach, *E. coli* genes were analyzed for their expression profile against the treatment response of plasma-activated PBS solution. The plasma-activated PBS solution chemistry is not yet fully understood. Based on our preliminary observations of generation of reactive oxygen species [3] [7] and the antioxidant mediated protection to *E. coli* cells (Figure 1), we thought of comparing with H_2O_2 treatment control. Looking at the large number of genes, only a change in expression of two-folds or more was considered for analysis. To minimize the number of false positives and enrich the transcriptomic response of differentially expressed genes, the signal intensities of >50 were considered (Figure 2(A)). The signals generated by plasma-activated PBS and H_2O_2 were normalized against untreated cells in PBS (as the cells were suspended in untreated PBS for 15 min; and likely to exhibit some starvation stress). On exposure to plasma-activated PBS and H_2O_2 , respectively 412 and 1272 genes differentially expressed, of which 218 were common among them when analyzed using pairwise comparison against untreated PBS condition (Figure 2(B)). Figure 3 is a heat map of the genes that are differentially expressed, and commonly involved in both, the plasma-activated PBS exposure and hydrogen peroxide exposure of cells.

Out of 412 genes differentially expressed upon plasma-activated PBS 230 were functionally defined genes, and rest 181 genes were either pseudo genes or hypothetical genes whose functional annotation is not defined. Total 120 genes were upregulated and 111 genes were downregulated. The findings of microarray assay of top

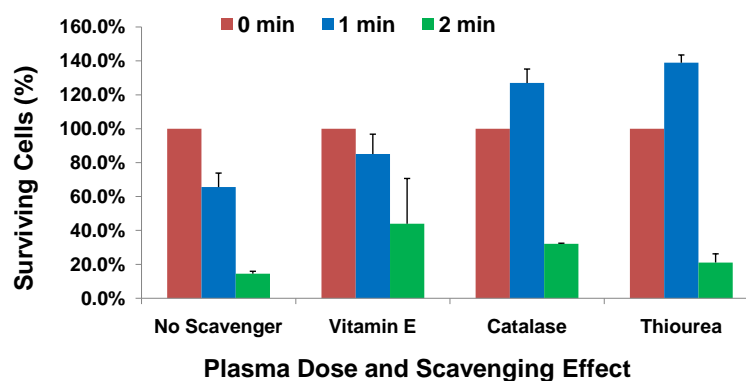


Figure 1. The kinetics of *E. coli* cell inactivation and the oxidative stress generated by plasma-treated PBS. A graphical presentation of XTT assay showing survival responses of *E. coli* against different plasma treatment time (at contact/holding time of 15 min), and the scavengers of reactive oxygen species (ROS) giving significant protection from plasma-activated PBS mediated inactivation. $*P \leq 0.05$ against corresponding “no scavenger” conditions; $n = 3$.

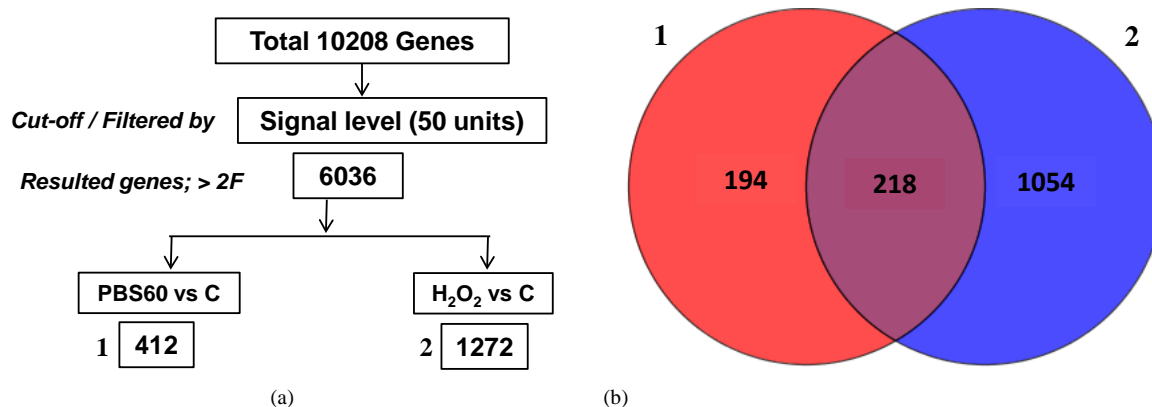


Figure 2. A schema of differentially expressed genes filtration that has >2 fold change (a) was adopted to reflect an enhancement of gene expression. A cut-off filtration of microarray fluorescing signals (50 units) was used to avoid false-positivity, which concurrently enhanced the gene expression profile; (b) A schematic presentation of differentially expressed genes of *E. coli* when cells exposed to plasma-activated PBS (PBS60) versus untreated PBS (circle 1; red), and cells exposed H_2O_2 versus untreated PBS (circle 2; blue). The 218 genes were differentially expressed, common in cells treated with H_2O_2 solution and plasma-activated PBS solution (PBS60), and were responsive to oxidative stress.

125 genes with their functional annotations which exhibited differential expression are grouped together and showed in **Table 1**. The transcriptomic response is categorized upon their cellular function, such as oxidative and other stress-related damage response, SOS response, nitrosative stress, cell envelop-related response, cell cycle/regulation, metabolism, transporter and Fe-S cluster assembly/cysteine synthesis. Differentially expressed genes represented about 4% of all genes of *E. coli* on chip. The fold increase is relative to untreated control. The upregulated genes were mostly related to stress response, iron-sulfur (Fe-S) cluster formation, cysteine biosynthesis pathway, biofilm formation, whereas downregulated genes were predominantly associated in general with cellular metabolism, ribosomal proteins, membrane proteins and porins, flagellum biosynthesis and motility.

3.3. SOS Response and Universal Distress Signals

SOS response is a global response to DNA damage wherein cell cycle is arrested and DNA repair (and/or mutagenesis) is induced, and includes the proteins related to Rec family (RecA, RecB, RecD, RecN). The SOS regulon involves protein RecA, responsible for inactivation of LexA repressor (and thus negatively regulated by LexA repressor protein dimmers), and is a complex of several genes that are coordinately expressed and involved in DNA repair. The RecA protein is under control of recombination regulator RecX; and RecN is required in DNA recombination. In present study, overall SOS genes were moderately upregulated (<2 folds; *recA*, *lexA*, *sulA*, *umuC*, *umuD*). Only the genes, *recN*, *nfo*, *nrdE* and *nrdF* were upregulated by >2 folds (**Table 1**).

3.4. Oxidative Stress Induced Genes

We observed highest expression of *oxyS* transcript (28.8 fold upregulation), an indicator of a generation of severe oxidative stress. Hydrogen peroxide detoxifying genes such as *katG* (catalase), *ahpC* (peroxiredoxin), *ahpF* (hydroperoxidase) and *yggP* (putative oxidoreductase) were differentially expressed; respectively 19.3, 3.9, 7.0, and 3.1 folds (**Table 1**). All these genes are responsive to H_2O_2 , suggests that hydrogen peroxide may be generated in *E. coli* cells. A transcriptional regulator of oxidative stress, SoxS was also upregulated (2.9 folds). SoxS is responsive to superoxide and singlet oxygen species. Out of three superoxide dismutases (SodA, MnSOD; SodB, FeSOD; and SodC, Cu/ZnSOD) that catalyze superoxide-like species, only transcript of *sodA* was upregulated. Other genes such as *grxA* (glutaredoxin-1) and *trxC* (thioredoxin-2) were also upregulated.

3.5. Other Stress-Related Response and Metabolism and Regulatory Genes

Reactive nitrogen species [13]—responsive genes such as *hda*, *atpA*, *gapA*, *tufA*, and *napA* were downregulated, whereas the genes of most of the Fe-S cluster assembly and cysteine synthesis pathway were significantly upregulated (**Table 1**). Genes, *cadC* and *aceF*, were upregulated by <2 folds, indicating that RNA mediated stress

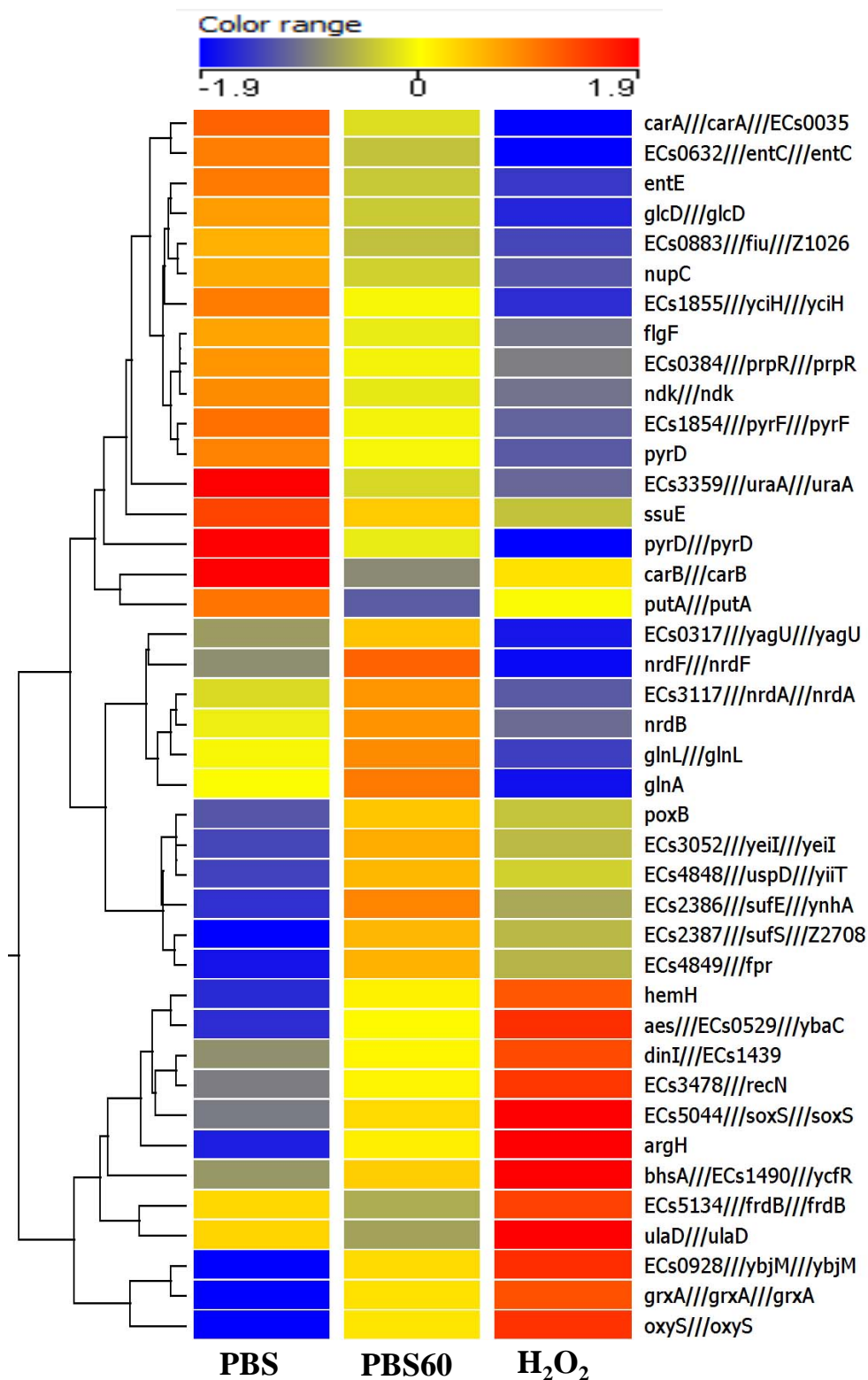


Figure 3. Hierarchical map of a comparison showing common genes that are differentially expressed in *E. coli* during exposure to plasma-activated PBS (PBS60) or H₂O₂ (oxidant; positive control), and compared with untreated PBS (PBS; negative control). Top 41 differentially expressed genes are shown here whose functional annotations are known. The functional annotation of most commonly influenced genes is shown in [Table 1](#).

Table 1. Functional analysis and category of differentially expressed genes involved in the response to plasma-activated PBS solution.

Gene	Fold expression	Regulation	Function
Oxidative damage response			
<i>oxyS</i>	28.8	Up	Small regulatory RNA, responsive to oxidative stress
<i>katG</i>	19.3	Up	Catalase/hydrogen peroxidase
<i>sodA</i>	2.1	Up	Superoxide dismutase/detoxification of superoxide radicals
<i>soxS</i>	2.9	Up	Transcriptional regulator of oxidative stress
<i>yhjA</i>	2.0	Up	Cytochrome-C peroxidase activity
<i>ahpF</i>	7.0	Up	Disulfide oxidoreductase activity/peroxidase
<i>ahpC</i>	3.9	Up	Peroxidase activity/peroxiredoxin
<i>ybiX</i>	-2.4	Down	Fe(II)-dependent oxygenase, hydroxylase activity
SOS response			
<i>recN</i>	2.4	Up	DNA recombination & repair
<i>dinL</i>	2.1	Up	DNA-damage-inducible protein
<i>recD</i>	2.3	Up	DNA repair, helicase activity, exonuclease V subunit
<i>recB</i>	2.1	Up	Exonuclease V subunit (recBCD complex)
<i>nfo</i>	4.4	Up	Endonuclease IV, DNA repair
Nitrosative stress			
<i>hda</i>	-3.2	Down	DNA replication initiation factor
<i>napA</i>	-2.6	Down	Nitrate reductase, periplasmic
<i>napD</i>	-3.2	Down	Assembly protein for periplasmic nitrate reductase
<i>napF</i>	-3.5	Down	Ferredoxin-type protein, Fe-Fe binding, electron transport
Related to cell envelop			
<i>tsx</i>	-2.5	Down	Porin activity, iron membrane-transporter
<i>ynbA</i>	2.2	Up	Inner membrane, phosphotransferase
<i>yjcH</i>	-2.2	Down	Inner membrane protein, DUF485 family
<i>yagU</i>	2.7	Up	Response to acidity, DUF1440 family
<i>yliE</i>	2.6	Up	Hydrolase activity, inner membrane protein
<i>flgF</i>	-2.0	Down	Flagellar/motility, flagellum basal body
<i>ycfS</i>	-3.5	Down	Peptidoglycan synthetase activity, cell shape
<i>livJ</i>	-2.8	Down	Carbon starvation induced gene, cell wall
Other stress-related response			
<i>sufA</i>	2.4	Up	Response to oxidative stress, Fe-S cluster protein
<i>acnA</i>	2.2	Up	Aconitate hydratase/oxidative stress response/TCA cycle
<i>uspD</i>	5.1	Up	Response to stress, cytoplasmic protein
<i>cstA</i>	-3.2	Down	Response to stress, plasma membrane protein

Continued

<i>astD</i>	-2.7	Down	Succinylglutamic semialdehyde dehydrogenase, response to stress
<i>bhsA</i>	-2.6	Down	Biofilm, cell surface and signaling protein, response to stress
<i>ymgC</i>	2.3	Up	Biofilm formation, predicted protein
<i>yhcN</i>	-2.7	Down	Cellular response to hydrogen peroxide, periplasmic space
<i>ycfR</i>	-2.6	Down	Periplasmic protein, biofilm formation, response to stress
Regulation/cell cycle/cell division			
<i>glnL</i>	2.1	Up	Histidine kinase activity, nitrogen regulation protein NR(II)
<i>ycfS</i>	-3.5	Down	Transferase, peptidoglycan synthetase, transcriptional regulator
<i>malT</i>	-3.3	Down	Transcriptional regulator MalT
<i>napD</i>	-3.2	Down	Assembly protein for periplasmic nitrate reductase
Metabolism			
<i>astA</i>	-2.1	Down	Acyltransferase, arginine N-succinyltransferase activity
<i>prpR</i>	-2.1	Down	Regulator for prp operon, propionate catabolism
<i>hcaR</i>	-2.0	Down	DNA-binding transcriptional regulator HcaR
<i>LipA</i>	2.0	Up	Lipoate synthase, transferase activity
<i>CarB</i>	-14.5	Down	Arginine biosynthetic process, synthase activity
<i>putA</i>	-6.6	Down	Proline dehydrogenase transcription
<i>pyrD</i>	-6.2	Down	Dihydroorotate dehydrogenase/UMP biosynthetic process
<i>argH</i>	4.6	Up	Argininosuccinate lyase activity
<i>poxB</i>	4.1	Up	Pyruvate oxidase, thiamine-dependent, FAD-binding
<i>hemH</i>	4.1	Up	Porphyrin biosynthetic process, ferrochelatase
<i>purD</i>	-3.5	Down	Purine base biosynthetic process, synthase
<i>ycfS</i>	-3.5	Down	Peptidoglycan synthetase activity
<i>carA</i>	-3.3	Down	Glutamine amidotransferase, synthase activity
<i>entC</i>	-3.3	Down	Isochorismate synthase, enterobactin biosynthesis
<i>entE</i>	-3.2	Down	Enterobactin synthase, siderophore biosynthesis
<i>pyrB</i>	-3.1	Down	Aspartate carbamoyltransferase activity
<i>leuB</i>	-3.1	Down	Isopropylmalate dehydrogenase, amino acid synthesis
<i>Pyrl</i>	-2.7	Down	Transferase activity, pyrimidine biosynthesis
<i>mdoD</i>	-2.1	Down	Osmoregulated glucan (OPG) biosynthesis, periplasmic protein
<i>alaC</i>	2.1	Up	Valine-pyruvate aminotransferase activity
<i>yggF</i>	-2.1	Down	Fructose 1,6 bisphosphatase, glycerol metabolism
<i>aes</i>	3.9	Up	Acetyl esterase, carboxylesterase activity
<i>rffH</i>	-2.2	Down	Extracellular polysaccharide biosynthetic process
<i>rffA</i>	-2.2	Down	Lipopolysaccharide biosynthetic process
<i>arnA</i>	2.0	Up	Decarboxylase, lipid biosynthetic process

Continued

<i>mdoD</i>	-2.1	Down	Osmoregulated glucan (OPG) biosynthesis
<i>gabD</i>	-2.4	Down	Succinate-semialdehyde dehydrogenase activity
<i>fumC</i>	2.4	Up	Fumarate hydratase, TCA cycle
<i>htrA</i>	-2.2	Down	Serine endoprotease, proteolysis
<i>pncB</i>	2.2	Up	Transferase activity, NAD biosynthetic process
Fe-S cluster assembly/cysteine synthesis			
<i>yfaE</i>	2.1	Up	Ferredoxin metabolic process, 2Fe-2S ferredoxin
<i>ygfT</i>	2.6	Up	Glutamate biosynthetic process, oxidoreductase, Fe-S subunit
<i>sufA</i>	2.4	Up	Iron-sulfur cluster assembly scaffold protein
<i>frdB</i>	-2.1	Down	Fumarate reductase, Fe-S subunit
<i>hypC</i>	-2.0	Down	Hydrogenase assembly chaperone
<i>grxA</i>	10.8	Up	Glutaredoxin 1, electron carrier activity
<i>sufD</i>	7.5	Up	Cysteine desulfurase activator complex subunit SufD
<i>sufC</i>	5.5	Up	Cysteine desulfurase ATPase component, ABC superfamily
<i>sufS</i>	8.4	Up	Bifunctional cysteine desulfurase
<i>Fpr</i>	7.0	Up	Ferredoxin-NADP reductase
<i>sufE</i>	3.5	Up	Cysteine desulfuration protein SufE, S-acceptor
<i>sufB</i>	3.7	Up	Iron-sulfur cluster assembly, SufBCD complex
<i>fhuC</i>	-2.1	Down	Iron-hydroxamate transporter subunit
<i>trxC</i>	6.1	Up	Thioredoxin-2
Transporters			
<i>mntH</i>	4.4	Up	Manganese ion transmembrane transporter activity
<i>fepA</i>	-2.1	Down	Iron-enterobactin outer membrane transporter
<i>ftnB</i>	-2.2	Down	Ferritin-like protein, cellular iron ion homeostasis
<i>lsrC</i>	2.1	Up	Transport system permease protein
<i>argH</i>	4.6	Up	Cellular amino acid biosynthetic process
<i>glnA</i>	2.3	Up	Glutamine synthetase & transport regulation, N ₂ -deprivation
<i>putP</i>	-3.7	Down	Major sodium/proline symporter
<i>cmtA</i>	2.6	Up	Carbohydrate transport, sugar:hydrogen symporter
<i>mngA</i>	2.4	Up	Carbohydrate transporter, IIABC components system
<i>malK</i>	2.0	Up	Maltose/maltodextrin transporter
<i>mdlB</i>	2.3	Up	Multidrug transporter
<i>nmpC</i>	-4.8	Down	Outer membrane porin protein, nmpC
<i>dppC</i>	-2.4	Down	Dipeptide/heme transporter
<i>narU</i>	5.1	Up	Nitrate/nitrite transporter protein
<i>araE</i>	2.3	Up	Arabinose-proton symporter, carbohydrate transporter
<i>dctA</i>	-3.4	Down	Sodium:dicarboxylate symporter activity

was less pronounced. Other stress-responsive universal genes which were differentially expressed include *acnA* (TCA cycle), *uspD* (cytoplasmic protein), *cstA* (plasma membrane protein), and *yhcN* (periplasmic space protein). The gene *bhsA* (formerly *ycfR*) that mediate biofilm formation was downregulated. Similarly, most of the genes encoding the proteins from biosynthesis and metabolic pathways were significantly downregulated (**Table 1**), and indicated that cells under (ROS or RNS) stress-induced adverse condition, indicating a general energy depletion, and conversely minimized energy consumption in order to prioritize survival. The expression of transcripts of *ycfS* (peptidoglycan synthetase), *malT* (MalT system), and *napD* (nitrate reductase) were significantly downregulated.

3.6. Cell Envelop and Membrane Associated and Transport Associated Genes

The transporter of manganese (*mntH*) was upregulated >4 folds, while almost all iron mediating transporters and related protein transcription, such as *fepA* (iron-enterobactin) *fnbB* (ferritin-like protein involved in cellular iron ion homeostasis), *fepC* (iron-enterobactin, ATP-binding subunit), *dppC* (dipeptide, heme transporter), *napF* (ferredoxin type protein) were downregulated. This suggests a disrupted iron homeostasis. The transporters of glucarate, glutamine, sugar:hydrogen, carbohydrate, maltodextrin, multidrug, amino acid, nitrate-nitrite were all upregulated (**Table 1**). The genes of outer membrane porin, *nmpC*, and iron membrane porin activity protein, *tsx* were downregulated.

4. Discussion

Despite of increasing reports on application of nonthermal plasma in disinfection and bacterial inactivation, relatively very little is known about the stress response of bacterial cell to it. In this study, we report gene expression profile and transcriptomic responses of *E. coli* to plasma-activated PBS solution which inactivates bacterial cell [7]. Three minutes plasma-treated PBS solution completely inactivated *E. coli* upon contact time of 15 minutes [7], and therefore not shown here. On the basis of our earlier observations, and the specific protection provided by ROS scavengers (**Figure 1**), we anticipated that this solution would mount oxidative stress in bacterial cell, and might be containing a mixture of ROS, including a stabilized species such as hydrogen peroxide [7] [8]. The amount of H₂O₂ generated in treated PBS at 60 seconds of plasma-activated of PBS was much smaller (0.42 mM) as compared to originally estimated 0.01% of H₂O₂ standard solution which corresponded to 3.26 mM. Hydrogen peroxide is a known antimicrobial agent. However, a concentration of H₂O₂ required to complete inactivation of most bacteria is as low as 124 mM (0.38%). Therefore, results suggest indirectly that other reactive species in the solution were present which participate in the inactivation process (either as added or synergistic effect) [7] [8]. Hence it is likely that oxidative stress mediating transcriptional response by plasma-activated PBS though not completely, would be little different as compared to H₂O₂ in type and intensity.

An analysis using global gene expression approach is recently used by Dr. Pruden's Laboratory [6] using argon gas plasma (an entirely different type of plasma set up, and parameters), where an overlapping expression patterns were observed; therefore this study becomes more interesting. Our microarray data analysis suggests that selection of hydrogen peroxide as a positive control for this study was logical. Hydrogen peroxide is known to generate reactive oxygen species and activate related pathways in *E. coli* [14].

4.1. SOS Response and Distress Signals

The SOS responses in *E. coli* are induced after DNA damage, and are dynamically regulated by interplay between Rec family protein, such as RecA, Lex protein, LexA, and Sul protein, Sula. RecN is a conserved SMC-like (structural maintenance of chromosomes) nucleoid-associated ATPase involved in the tethering of chromatids and in double strand break (DSB) repairs [15], and thus involved in DNA recombination and repair. RecN plays a crucial role in homologous recombination-dependent DSB repair, and it is required upstream of RecA-mediated strand exchange. RecN is also highly conserved in *E. coli* and it has two SOS boxes in its promoter region. Therefore, the expression of *recN* is tightly regulated by LexA repressor [16]. In *E. coli*, DSB repair is initiated by RecBCD, a main double-strand exonuclease. The transcription of *recN*, *recB* and *recD* components was increased in the present study. Operon *nrdAB* and *nrdEF* encoding ribonucleotide reductases which supply deoxynucleoside triphosphate (dNTP) substrates for DNA replication were found upregulated (**Table 1**). Endonuclease IV encoded by *nfo* is also involved in DNA repair, and is a component of SoxRS regulon system, and

activated by SoxS superoxide-responsive regulatory protein. Thus SOS response and oxidative stress are reportedly related [17].

4.2. Oxidative Stress Response Is Predominant

E. coli has several major regulators that are activated during oxidative stress and undergo conformational changes, but two regulon systems are important in oxidative stress management. These are OxyR and SoxR transcriptional regulators, sensitive to oxidation in presence of hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) respectively and subsequently regulate the expression of an array of cognate genes. ROS are the inevitable by-product of oxidative stress and oxidation, and the species such as H_2O_2 and O_2^- are generated when they react with flavin cofactors containing dehydrogenases and other cell components. Reactive nitric oxide is also generated as denitrification intermediate, and can further react with superoxide or hydroxyl ($\cdot\text{OH}$) radicals. Reactive $\cdot\text{OH}$ often results from H_2O_2 interaction with unincorporated ferrous iron by Fenton chemistry [18]. OxyS is a small regulatory RNA, responsive to oxidative stress, especially H_2O_2 rich environment where it detoxifies the latter. We observed highest expression of *oxyS* transcript (Table 1), an indicator of a generation of severe oxidative stress, and may suggest a synergy of different reactive oxidative species that enhance inactivation of *E. coli* cells during plasma-activated PBS exposure. Similar observation was noted by Pruden Laboratory [6] during direct argon gas plasma application, indicating that oxidative stress is observed in both, direct plasma exposure and plasma-activated fluid. OxyS further regulates multiple targets (over a dozen genes); of which *sodA* is one of the major players that was upregulated in our study. Sox regulon is also closely related with Mar regulon genes, but we did not see any differential regulation of the latter in our study. SoxR has been shown to detect superoxide via oxidation of its iron-sulfur cluster, and OxyR detects H_2O_2 via oxidation of specific cysteine residues. The important members of OxyR-regulated genes, such as *ahpC*, *ahpF*, *katG*, *grxA*, *trxC* and *gor* were upregulated both in the conditions of plasma-treated PBS solution and hydrogen peroxide control. Together these findings suggest the generation of reactive oxygen species, such as hydrogen peroxide and superoxide.

4.3. Other Stress-Related Genes and Their Metabolic Responses

Cells under (ROS or RNS) stress often activate transcription factor OxyR which in turn regulates the expression of a large panel of genes, including a small regulatory RNA, *oxyS*, as mentioned above. Such observations are reported during severe oxidative as well as nitrosative stress conditions [19] [20], and in argon gas plasma system [3] [6]. We observed reactive nitrogen species-responsive genes [13] such as *hda* and *napA* were downregulated, and the genes involved in Fe-S cluster assembly and sulfur acquisition were significantly upregulated. The *hda* encode for DNA replication initiation factor, and is the first *E. coli* chromosomal gene shown to initiate translation with CTG. Nap enzyme complex is localized to the periplasm with NapA, NapB and NapC, and identified as the essential components for nitrate reduction in *E. coli*. NapA is enzymatically active subunit that contains Fe-S cluster and molybdenum co-factor; reduces NO_3 to NO_2 . RNS function as powerful antimicrobials in both the mammalian host cell and bacterial cells [13]. Bacteria developed certain mechanisms to survive under nitrosative stress by the mechanisms such as RNS-mediated protein modifications, reversible binding of nitric oxide to multiple Fe-S clusters or thiol groups [21]. More than 110 proteins in *E. coli* require Fe/S clusters to function, where they control enzyme catalysis, electron transport, and regulation of gene expression. During *in-vitro*, large amounts of free iron and sulfide become available spontaneously in assembly of Fe/S clusters, however *in-vivo* these elements are toxic to cells, and hence concentrations are tightly regulated [22]. Bacteria utilize at least one of the three systems known to assemble such clusters, namely Isc, Suf and Nif systems, of which former two (mediated by *isc* and *suf* operons) are reportedly induced under ROS/RNS mediated oxidative stress conditions. The *suf* (*sufABCDSE*) operon encoding a Fe-S assembly system is induced by peroxides through activators OxyR and IscR in *E. coli* [23]-[25], and is an alternative system (to Isc) under oxidative and Fe-limiting conditions. In the present study only Suf system was found differentially expressed, and indicates that plasma-activated PBS probably produce peroxides in sufficient amount, required for activation of *suf* operon. Our earlier findings on exposure of *E. coli* cells to direct plasma discharge (not through plasma-treated fluid) also demonstrated the generation of hydrogen peroxide, and the specific scavenger such as catalase was able to decompose peroxide significantly [3]. The present microarray analysis is therefore important in this context, and further studies are required for comparing the exact differences.

4.4. Plasma-Treated PBS Solution Influence Cell Envelop and Membrane Associated Mechanisms

It can be predicted that the interaction of ROS generated by plasma-treated solution exposure to cellular iron may have potential detrimental effect on bacteria, either by unavailability of iron for bacterial cell (iron starvation) or leading to series of lethal reactions that further regenerate hydroxyl radical (such as Fenton reaction) [26]-[28], influencing the expression of several important functional genes. The genes encoding most carbohydrate/sugar transporters were upregulated, whereas the ones for biosynthesis were downregulated. Plasma-activated PBS solution had downregulated genes involved in flagella, motility, fimbriae, adhesin, and quorum sensing (Table 1). Bacterial biofilm formation and cell motility are intrinsically correlated [29], and both compromised by oxidative stress, acid stress or starvation [30], and differentially expressed, depending upon the amount of stress. A transpeptidase, the protein (encoded by *ycfS*) required for peptidoglycan synthesis was down-regulated during plasma-treated PBS, and indicates that the energy involving processes are minimized by *E. coli* under such stress. In addition to ROS/RNS generated in plasma-treated PBS, the solution became acidic [7]. We demonstrated earlier that antimicrobial property is not due to a mere change in pH but a likely synergy effect of all reactive species and concurrent reduction in pH. Acid-induced genes are associated with increased catabolic processes, generate oxidative stress, activate heat shock regulons, and differentially regulate large number of proton gradient, periplasmic and cell envelope proteins, energy-driven synthesis and assembly of flagella.

Under oxidative stress in *E. coli* several transporters are differentially expressed such as iron transporters. Iron is required for many metabolic processes and plays a role in protection against oxidative damage. However, excess iron levels in cells contribute to oxidative damage through the generation of free radicals [18]. Global regulators such as OxyR and SoxR have an association with their co-repressor that represses transcription of genes encoding high-affinity transport systems and other related proteins involved in iron metabolism, and thus tightly control iron homeostasis. We observed a downregulation of genes that encodes iron transporting and binding proteins, such as *tsx* (iron membrane transporter exhibit porin like activity), *fepA* (iron-enterobactin outer membrane transporter), *fhuC* (iron-hydroxamate transporter subunit), *fthB* (ferritin-like protein having iron ion homeostasis) and *fepC* (the ATP-binding subunit of iron-enterobactin transporter (Table 1). In contrary, manganese ion transporter encoded by *mntH*, and manganese-dependent superoxide dismutase encoded by *sodA* were found upregulated. Thus control of both, the iron and manganese transport, and Fe- and Mn-dependent defense against oxidative stress could be well coordinated [31]. Further details of the mechanisms are required to be investigated.

5. Conclusion

This transcriptomic study suggests that *E. coli* cells differentially express several important genes that are responsive to oxidative stress generated upon exposure to plasma-activated PBS solution. The genes responsive to hydrogen peroxide, superoxide and singlet oxygen, and reactive nitrogen species were observed, and might be collectively exerting their damaging effect on *E. coli* cell. The data presented here are predictive and for the guidance, and further detailed studies would be interesting to elucidate the exact nature of responses to different nonthermal plasma set-ups and their various parameters.

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Competing Interest

The authors declare “no conflict” and no competing interests.

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