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GENOMICS AND PROTEOMICS

Comparative global transcription analysis of sodium hypochlorite, peracetic acid, and hydrogen peroxide on *Pseudomonas aeruginosa*

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Abstract Disinfectants are routinely used in hospitals and health care facilities for surface sterilization. However, the mechanisms by which these disinfectants kill and the extent to which bacteria, including *Pseudomonas aeruginosa*, are resistant remains unclear. Consequently, *P. aeruginosa* nosocomial infections result in considerable casualties and economic hardship. Previously, DNA microarrays were utilized to analyze the genome-wide transcription changes in *P. aeruginosa* after oxidative antimicrobial (sodium hypochlorite, peracetic acid, and hydrogen peroxide) exposure. Simultaneous analysis of these transcriptome datasets provided a comprehensive understanding of the differential responses to these disinfectants. An analysis of variance, functional classification analysis, metabolic pathway analysis, Venn diagram analysis, and principal component analysis revealed that sodium hypochlorite exposure resulted in more genome-wide changes than either peracetic acid or hydrogen peroxide exposures.

Keywords *Pseudomonas aeruginosa* · Sodium hypochlorite · Peracetic acid · Hydrogen peroxide · Microarrays

Pseudomonas aeruginosa PA01 (*P. aeruginosa*) is an opportunistic pathogen that infects organisms and causes nosocomial infections (Vincent et al. 2004). These infections are more common in patients with defective immune systems such as cystic fibrosis and can be lethal to those suffering from compromised immune systems (Tummler and Kiewitz 1999). *P. aeruginosa* infections may also occur in cancer patients, patients suffering from urinary tract infections, and patients suffering from burn wounds (Bodey et al. 1983). *P. aeruginosa* is well suited to survive in a wide variety of environments (water, soil, and animals) and is prevalent in common everyday surroundings (Fink 1993). Infections caused by *P. aeruginosa* are often difficult to treat due to the prominent resistance exhibited by the pathogen to antimicrobial agents (Hancock 1998).

Oxygen exposure, and in particular the elicitation of reactive oxygen species, can result in DNA and lipid damage that display antimicrobial effects. Indeed, oxidative antimicrobials (sodium hypochlorite, peracetic acid, hydrogen peroxide, etc.) have commonly been used to eliminate pathogenic bacteria, such as *P. aeruginosa* (Kitis 2004; Spoering and Lewis 2001). Hydrogen peroxide is widely used as a disinfectant in the concentration ranges of 3–90% (*v*/*v*) and is considered environmentally friendly because it breaks down into water and oxygen (Block 2001). Acute hydrogen peroxide exposure to bacterial cells generates hydroxyl free radicals (·OH) that attack DNA resulting in the cellular death (Carlsson and Carpenter 1980; Demple

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and Halbrook 1983). Sulfhydryl groups and disulfide bonds are suspected to be targeted by these free radicals as well (Block 2001). Peracetic acid is often considered a more potent disinfectant than hydrogen peroxide as it is effective at concentrations <0.3% (*v*/*v*) (Block 2001). Peracetic acid is also considered environmentally friendly as it decomposes to acetic acid and oxygen but has added advantages over hydrogen peroxide as it is not susceptible to peroxidases and retains its activity in the presence of organic loads (Malchesky 1993). Peracetic acid's mechanism of action is speculated to be the denaturation of proteins and enzymes and increased cell wall permeability by oxidizing sulfhydryl and disulfide bonds (Block 2001). Sodium hypochlorite is the most widely used disinfectant; comprising the active ingredient in more than 350 registered products by the US Environmental Protection Agency. In aqueous solutions, sodium hypochlorite ionizes into Na^+ and OCl^- and establishes an equilibrium between these ions and hypochlorous acid (HOCl; Bloomfield 1996). The HOCl moiety is dominant in the pH 4–7 range, and the OCl^- moiety is dominant above pH 9 (White 1986). Hypochlorous acid has been shown to disrupt oxidative phosphorylation (McDonnell and Russell 1999), however, the precise mechanism of action by which sodium hypochlorite kills remains to be elucidated (Rutala 1996).

Transcriptome analyses of bacteria treated with these antimicrobials have only recently emerged, and no comparative analysis between different antimicrobials has been made (Chang et al. 2005a, b, 2006; Palma et al. 2004; Zheng et al. 2001). Notably, microarray studies of a bacterial response to hydrogen peroxide have appeared for many species of bacteria, including the following: *Bacillus subtilis* (Hayashi et al. 2005), *Staphylococcus aureus* (Chang et al. 2006), and *Streptococcus pneumoniae* (Ulijasz et al. 2004). Our recent studies of the response of *P. aeruginosa* to peracetic acid and sodium hypochlorite are the only transcriptome analysis concerning these antimicrobials among Eubacteria (Chang et al. 2005b; Small et al. 2007). There have been many reports attempting to compare microarray studies, either for evaluating the responses of an organism to a series of treatments or perturbations or responses of many organisms to the same treatment or perturbation. Most often, these results are indecisive; indeed, there are many reports providing guidance on how one treats data from disjointed microarray studies to statistically glean new insight. This study, comparing three independent transcriptome reports, takes advantage of the almost identical experimental conditions underpinning the investigations. In particular, the real-time polymerase chain reaction (PCR)-validated genome-wide changes in *P. aeruginosa* (based on data from Affymetrix *Pseudomonas* GeneChip arrays) after a 20-min sublethal exposure to sodium hypochlorite, peracetic acid, and hydrogen peroxide were investigated using several formats including one-way analysis of variance (ANOVA), Venn diagrams, and principal component analysis (PCA). The results suggest that the mechanisms by which peracetic acid, hydrogen peroxide, and sodium hypochlorite act upon cell metabolism are clearly distinct. Sodium hypochlorite is noted for multiple targets of somewhat distant function, which might be a basis for its unparalleled effectiveness (Chang et al. 2005a, b). This is the first comparative global gene transcription analysis of these three oxidative stressors. We believe this study and further comparative reports will help elucidate the mechanisms by which disinfectants kill microbes and facilitate the design of more effective antimicrobials, including those comprising mixtures of various compounds.

Materials and methods

Cells and media, sampling, and microarray procedures

In this study, we used *P. aeruginosa* PA01 obtained from Dr. E. Peter Greenberg's laboratory at the University of Iowa. To maintain homogeneous culture samples throughout our experiments, we employed the following three steps: (1) we initiated *P. aeruginosa* cultures at 37°C with shaking at 250 rpm using sterilized Luria–Biertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter); (2) after 17 h, we diluted the overnight cultures 1:100 in prewarmed LB broth and incubated at 37°C with shaking at 250 rpm until an optical density at 600 nm (OD_{-600-}) reached the early logarithmic phase (~0.8); and (3) we rediluted the cells 1:10 in prewarmed LB broth and incubated at 37°C with shaking at 250 rpm (Chang et al. 2005a, b). RNA for microarray analysis was isolated 20 min after the addition of water (for the control samples), 4.4 mM sodium hypochlorite, 1 mM hydrogen peroxide, or 0.5 mM peracetic acid (Chang et al. 2005a, b; Small et al. 2007). Complementary DNA was synthesized from 12 μg of RNA according to the standard protocols for the Affymetrix *P. aeruginosa* GeneChip array (Affymetrix). Hybridization and scanning of the arrays were performed according to the standard protocols for the Affymetrix *P. aeruginosa* GeneChip array (Affymetrix).

Microarray data analysis

Data analysis was performed with the Affymetrix GeneChip Operating Software (GCOS) v. 1.0 and GeneSpring GX v. 7.3 (Agilent Technologies). The following parameters were employed for GCOS expression analysis: *alpha* 1, 0.04; *alpha* 2, 0.06; *tau*, 0.015; target signal, 150. Four sodium hypochlorite-exposed sample replicates, four peracetic acid-exposed sample replicates, and four hydro-

gen peroxide-exposed sample replicates were then normalized to the four water-diluted (control) sample replicates. A master present/marginal list of present/marginal call genes was created by merging the lists of genes that received present/marginal calls from 50% or more of the replicates for each sample set (four water-diluted control samples, four sodium hypochlorite-treated cultures, four peracetic acid-treated cultures, and four hydrogen peroxide-treated cultures). Gene expression changes with statistical significance were identified by 1-way ANOVA (p cutoff value, 0.05). Fold change was then calculated as the log transformed (median log-of-ratio) between the signals of the four water-diluted control samples and to each of the following with respect to the exposed disinfectant as follows: four 4.4 mM sodium hypochlorite-treated cultures, four 0.5 mM peracetic acid-treated cultures, and four 1 mM hydrogen peroxide-treated cultures. The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE7402.

Results

P. aeruginosa transcriptome changes in response to oxidative stress

A sublethal exposure of 4.4 mM (0.03%) sodium hypochlorite (Small et al. 2007), 0.5 mM peracetic acid (Chang et al. 2005b), and 1 mM hydrogen peroxide (Chang et al. 2005a) for 20 min showed inhibition of growth. Notably, the concentrations of stressors for study were selected so that the attenuation in cell growth was fairly severe to ensure a response (i.e., approximately 50% of the initial growth rate), while still enabling harvest of quality messenger RNA (mRNA) for microarray analysis. From the 5,570 genes in the *P. aeruginosa* genome, 5,237 genes passed the present/marginal restriction described above to form the master present/marginal list. From the 5,237 genes in the master present-marginal list, 3,197 genes across all three treatments showed statistical significance based on the one-way ANOVA (sodium hypochlorite had 1,245 upregulated and 1,952 downregulated genes, peracetic acid had 1,209 upregulated and 1,988 downregulated genes, and hydrogen peroxide had 1,568 upregulated and 1,629 downregulated genes). However, as these groups are being compared to each other, a fold change threshold of twofold or more was established to differentiate responses. This statistical analytical analysis provided a unique, global method for comparing these treatments, as our previous studies simply used t tests comparing a set of treatment samples to the control samples. The one-way ANOVA analysis based on the treatment provides a more robust analysis to compare the treatments rather then just comparing individual t tests against one another. To visualize the union of the datasets, a Venn diagram analysis based on various fold change differences was performed on the statistically significant genes determined by the one-way ANOVA to further analyze logical relationships (unions and intersections) of genes between all the treatments. Based on these values, a conservative twofold or more upregulation or downregulation threshold was selected to differentiate the seven regions of the Venn diagram as shown in Fig. 1. Thus, from the 3,197 genes that showed statistical significance based on the one-way ANOVA, sodium hypochlorite had 457 upregulated and 625 downregulated genes, peracetic acid had 362 upregulated and 413 downregulated genes, and hydrogen peroxide had 187 upregulated and 204 downregulated genes at the twofold or more change threshold level.

Functional classifications analysis

Functional classifications of the responding genes are provided in Fig. 2. Not shown in these figures is the "hypothetical, unclassified, unknown" functional class, which comprised the greatest number of affected genes for all the disinfectant-exposed samples. Functional classes are taken from *P. aeruginosa* Community Annotation Project (Winsor et al. 2005). A Venn diagram analysis was performed on the functional classifications marked with asterisks in Fig. 2 to further analyze logical relationships (unions and intersections) of genes in each of these functional classes. The results are shown in Table 1 for the twofold or more increased genes and Table 2 for the twofold or more decreased genes (Table 2) and reference the regions defined in the Venn diagram (Fig. 1).

Metabolic pathway analysis

P. aeruginosa metabolic pathways were compared between the disinfectant-exposed samples and the (water-diluted) control samples based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; Ogata et al. 1999). These metabolic pathways were compiled into tables to organize the data based on metabolic functions. Table 3 shows the oxidative phosphorylation pathway genes, while Table 4 displays the Enteroff–Doudoroff (ED) pathway and Embden–Meyerhof–Parnas (EMP) pathway based on prior characterizations and genetic mappings (Roehl et al. 1983; Roehl and Phibbs 1982). Table 5 shows ATP-binding cassettes (ABC) for organic sulfur transport-related genes. Finally, Supplemental Tables 1, 2, and 3 display cellular protective mechanism-related genes, iron regulation-related genes, and pyocin system-related genes, respectively, which were organized

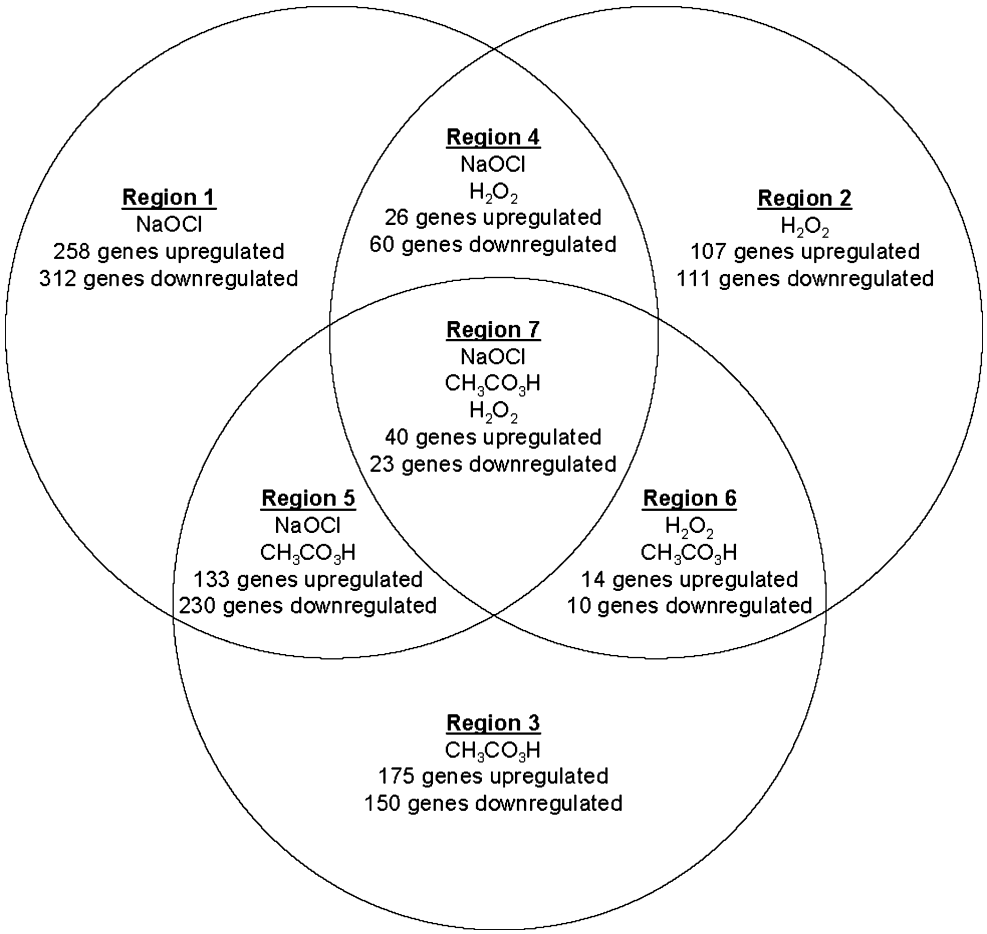

Fig. 1 This generic Venn diagram shows the unions and intersections of the three disinfectants. Regions are defined as follows: sodium hypochlorite exclusively is in *Region 1*, hydrogen peroxide exclusively is in *Region 2*, peracetic acid exclusively is in *Region 3*, the intersection of sodium hypochlorite and hydrogen peroxide is in *Region 4*, the intersection of sodium hypochlorite and peracetic acid is in *Region 5*, the intersection of hydrogen peroxide and peracetic acid is in *Region 6*, and the intersection of all three disinfectants is in *Region 7*. The regions defined in this diagram are referenced in Tables 1 and 2

based on our prior studies of peracetic acid and hydrogen peroxide (Chang et al. 2005a, b).

Principal component analysis

Finally, a PCA based on conditions was performed on the master present/marginal list (5,237 genes) for all of the conditions (four control group replicates, four sodium hypochlorite-exposed group replicates, four peracetic acid-exposed group replicates, and four hydrogen peroxide-exposed group replicates) using Genespring GX 7.0 centering. PCA reduces data dimensionality by reducing a complex data set to a lower dimension to reveal a more simplistic structure of the data (Daffertshofer et al. 2004). A PCA on conditions is typically used to show data integrity. The results of this analysis are shown in Fig. 4. Based on the 86.52% total variance calculated by the PCA on conditions analysis, PCA component 1 had an eigenvalue of 770.2 and 56.12% of the variance, PCA component 2 had an eigenvalue of 246.2 and 17.94% of the variance, and PCA component 3 had an eigenvalue of 171.0 and 12.46% of the variance. In PCA, large variances represent high signal-to-noise ratios (SNR) in the data that reveal the most interesting dynamics of the data set. Thus, PCA component 1 represented the most dynamic difference between the samples. While PCA component 2 and PCA component 3 both represented significantly less dynamic difference among the samples but still exceeded the SNR of the data set.

Discussion

Functional classification analysis

Based on the one-way ANOVA statistically significant twofold or more changes, sodium hypochlorite exposure resulted in more upregulated or downregulated genes than either hydrogen peroxide exposure or peracetic acid

Fig. 2 Function classification of the number of genes with statistically significant increased mRNA levels. The number of genes are shown for each functional classification based on a twofold or more increase or decrease in mRNA transcript level for 4.4 mM sodium hypochlorite (■), 0.5 mM peracetic acid (□), and 1 mM hydrogen peroxide (▒) after a 20-min exposure to each corresponding disinfectant. The unions and intersections of the functional classes marked with a *single asterisk* (downregulations) are further analyzed in Table 1 and the functional classes marked with a *double asterisk* (upregulations) are further analyzed in Table 2

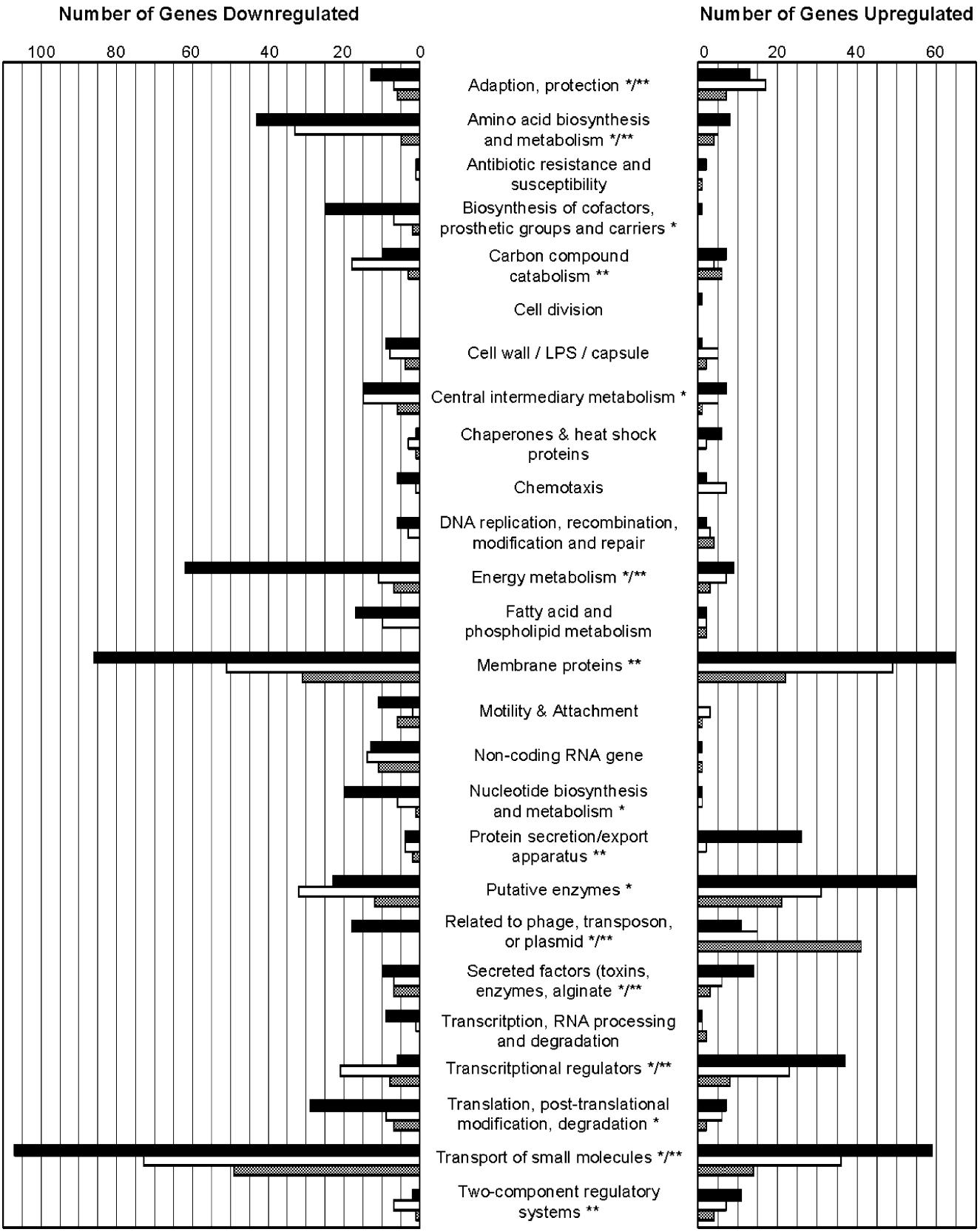
Number of Genes Downregulated
Number of Genes Upregulated
100
80
60
40
20
0
0
20
40
60
Adaption, protection */**
Amino acid biosynthesis and metabolism */**
Antibiotic resistance and susceptibility
Biosynthesis of cofactors, prosthetic groups and carriers *
Carbon compound catabolism **
Cell division
Cell wall / LPS / capsule
Central intermediary metabolism *
Chaperones & heat shock proteins
Chemotaxis
DNA replication, recombination, modification and repair
Energy metabolism */**
Fatty acid and phospholipid metabolism
Membrane proteins **
Motility & Attachment
Non-coding RNA gene
Nucleotide biosynthesis and metabolism *
Protein secretion/export apparatus **
Putative enzymes *
Related to phage, transposon, or plasmid */**
Secreted factors (toxins, enzymes, alginate */**
Transcritption, RNA processing and degradation
Transcritptional regulators */**
Translation, post-translational modification, degradation *
Transport of small molecules */**
Two-component regulatory systems **

Table 1 Venn diagram data for functional classifications of statistically significant twofold or more genes upregulated between sodium hypochlorite, peracetic acid, and hydrogen peroxide

Region	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Region 7
Disinfectant(s)	NaOCl	H_2O_2	CH_3CO_3H	NaOCl H_2O_2	NaOCl CH_3CO_3H	H_2O_2 CH_3CO_3H	NaOCl H_2O_2 CH_3CO_3H
Functional classification	Number of Genes						
Adaption, protection	4	5	9	1	7	0	1
Amino acid biosynthesis and metabolism	4	3	2	1	3	0	0
Carbon compound catabolism	6	5	4	1	0	0	0
Energy metabolism	6	2	4	0	2	0	1
Membrane proteins	33	8	21	5	19	1	8
Protein secretion and export apparatus	24	0	0	0	2	0	0
Putative enzymes	40	15	18	2	9	0	4
Related to phage, transposon, or plasmid	0	29	3	0	0	1	11
Secreted factors (toxins, enzymes, alginate)	11	3	3	0	3	0	0
Transcriptional regulators	25	6	12	1	10	0	1
Transport of small molecules	36	6	19	6	15	0	2
Two-component systems	8	1	4	1	1	1	1

exposure. This same comparative pattern was observed in the functional classification analysis. By visually observing Tables 1 and 2, four striking features stood out as follows: (1) sodium hypochlorite exposure resulted in more twofold or more downregulated genes in the "Energy Metabolism" functional class suggesting that sodium hypochlorite exposure may limit energy production by the cell more than peracetic acid or hydrogen peroxide exposure. (2) Exposure to sodium hypochlorite generally resulted in the downregulation of genes in the functional classes marked with a single asterisk in Fig. 2 and generally had more genes upregulated in the functional classes marked with a double asterisk in Fig. 2, which demonstrated that sodium hypochlorite exposure resulted in more genome-wide changes than peracetic acid or hydrogen peroxide exposure. (3) Exposure to hydrogen peroxide resulted in the fewest number of twofold or more upregulated and downregulated genes in the entire asterisk-marked functional classes, which suggested that hydrogen peroxide treatment resulted in fewer genome-wide changes than sodium hypochlorite or peracetic-acid exposure. (4) There were very few functional classes that were not affected by all treatments, and by far,

Table 2 Venn diagram data for functional classifications of statistically significant twofold or more genes downregulated between sodium hypochlorite, peracetic acid, and hydrogen peroxide

Region	Region 1	Region 2	Region 3	Region 4	Region 5	Region 6	Region 7
Disinfectant(s)	NaOCl	H_2O_2	CH_3CO_3H	NaOCl H_2O_2	NaOCl CH_3CO_3H	H_2O_2 CH_3CO_3H	NaOCl H_2O_2 CH_3CO_3H
Functional classification	Number of Genes						
Adaption, protection	8	4	4	2	3	0	0
Amino acid biosynthesis and metabolism	20	3	12	2	21	0	0
Biosynthesis of cofactors, prosthetic groups and carriers	21	2	3	0	4	0	0
Central intermediary metabolism	5	5	6	1	9	0	0
Energy metabolism	49	2	2	4	8	0	1
Fatty acid and phospholipid metabolism	11	0	4	0	6	0	0
Membrane proteins	35	11	10	13	34	3	4
Nucleotide biosynthesis and metabolism	16	0	3	1	3	0	0
Putative enzymes	7	6	19	3	10	0	3
Related to phage, transposon, or plasmid	18	0	3	0	0	0	0
Secreted factors (toxins, enzymes, alginate)	2	1	4	5	2	0	1
Transcriptional regulators	2	8	17	1	4	0	0
Translation, posttranslational modification, degradation	23	4	4	1	3	0	2
Transport of small molecules	31	17	15	21	47	3	8

Table 3 Comparison of genes related to oxidative phosphorylation for sodium hypochlorite-, peracetic acid-, and hydrogen peroxide-exposed *P. aeruginosa*

Gene number	Gene name	Gene description	NaOCl	CH_3CO_3H	H_2O_2
Complex I, NADH dehydrogenase I					
PA2637	*nuoA*	NADH dehydrogenase I chain A	−2.21	−1.69	−1.81
PA2638	*nuoB*	NADH dehydrogenase I chain B	−3.08	−2.35	−1.23
PA2639	*nuoD*	NADH dehydrogenase I chain C, D	−2.92	−2.02	−1.22
PA2640	*nuoE*	NADH dehydrogenase I chain E	−2.70	−1.80	+1.07
PA2641	*nuoF*	NADH dehydrogenase I chain F	−2.72	−1.78	−1.20
PA2642	*nuoG*	NADH dehydrogenase I chain G	−3.07	−1.69	+1.04
PA2643	*nuoH*	NADH dehydrogenase I chain H	−3.08	−1.58	−1.05
PA2644	*nuoI*	NADH dehydrogenase I chain I	−3.48	−1.89	1.00
PA2645	*nuoJ*	NADH dehydrogenase I chain J	−3.48	−1.93	−1.32
PA2646	*nuoK*	NADH dehydrogenase I chain K	−3.39	−1.99	−1.15
PA2647	*nuoL*	NADH dehydrogenase I chain L	−3.86	−1.95	−1.11
PA2648	*nuoM*	NADH dehydrogenase I chain M	−3.62	−1.96	−1.07
PA2649	*nuoN*	NADH dehydrogenase I chain N	−3.75	−1.73	−1.04
Complex II, fumarate reductase					
PA1581	*sdhC*	Fumarate reductase C subunit	−2.56	−1.68	−1.17
PA1582	*sdhD*	Fumarate reductase D subunit	−2.40	−1.43	−1.01
PA1583	*sdhA*	Fumarate reductase A subunit	−2.44	−1.30	−1.08
PA1584	*sdhB*	Fumarate reductase B subunit	−2.44	−1.39	−1.27
Complex III, cytochrome bc1 complex					
PA4131		Probable iron–sulfur protein	−17.79	−8.77	−2.22
PA4429		Probable cytochrome c1 precurson	−3.46	−1.73	−1.84
PA4430		Probable cytochrome b	−3.44	−1.95	−1.41
PA4431		Probable iron–sulfur protein	−2.28	−1.53	1.08
Complex IV, cytochrome c oxidase					
PA1317	*cyoA*	Ubiquinol oxidase subunit II	−5.59	+1.45	−2.63
PA1318	*cyoB*	Ubiquinol oxidase subunit I	−3.57	+1.03	−2.64
PA1319	*cyoC*	Ubiquinol oxidase subunit III	−4.12	+1.01	−2.09
PA1320	*cyoD*	Ubiquinol oxidase subunit IV	−5.29	+1.13	−2.05
PA1321	*cyoE*	Ubiquinol oxidase protein CyoE	−2.53	+1.08	−1.98
PA1552		Probable cytochrome c	−4.85	−1.90	−1.52
PA1553		Cytochrome c oxidase subunit	−4.63	−1.88	−1.38
PA1554		Subunit (cbb3-type)	−3.85	−2.23	−1.28
PA1555		Probable cytochrome c	−12.58	−2.46	−1.36
PA1556		Cytochrome c oxidase subunit	−14.47	−2.46	−1.94
PA1557		Cytochrome oxidase subunit	−21.65	−3.11	−1.91
PA4133		Subunit (cbb3-type)	−5.05	−4.50	−2.12

the most affected functional class was membrane proteins, particularly those associated with small molecule transport across the periplasmic or cytoplasmic membrane.

To further visualize the changes in these functional classes, a Venn diagram analysis of these functional classes marked with a single asterisk (downregulated) or a double asterisk (upregulated) revealed that sodium hypochlorite indeed does have more uniquely upregulated and downregulated genes for all of the functional classes marked with asterisks in Fig. 2 (see Tables 1 and 2), except in the "Related to phage, transposon, or plasmid" functional class, where the hydrogen peroxide exposure resulted in more than twice as many genes upregulated compared to sodium hypochlorite or peracetic acid exposure. The number of upregulated and downregulated genes resulting from peracetic acid exposure generally fell in between the highly affected sodium hypochlorite-exposed and the minimally affected hydrogen peroxide-exposed groups in either the overall twofold or more change analysis and the functional classification analysis. However, exposure to peracetic acid resulted in upregulation of the genes in the "Adaption, protection" functional class and downregulation of the genes in the "Transcriptional regulators" functional classification.

Oxidative phosphorylation and electron transport

The oxidative phosphorylation pathway analysis expanded upon the "Energy metabolism," "Membrane proteins," and "Putative enzymes" functional classes. The large downregulation of the "Energy metabolism" functional class after

Table 4 Comparison of Enteroff–Doudoroff pathway and Embden–Meyerhof–Parnas pathway gene expression for sodium hypochlorite-, peracetic acid-, and hydrogen peroxide-exposed *P. aeruginosa*

Gene number	Gene name	Gene description	NaOCl	CH_3CO_3H	H_2O_2
PA0337	*Pts*	Phosphotransferase system	+1.11	−1.39	+1.36
PA0555	*Fda*	Fructose-1,6-diphosphate aldolase	NSS	NSS	NSS
PA2262	*KguT*	Probable 2-ketogluconate transporter	−1.92	−1.10	+1.23
PA2265	*Gad*	Gluconate dehydrogenase	−1.88	+1.13	−1.16
PA2290	*Gcd*	Glucose dehydrogenase	−2.95	−1.12	−1.31
PA2321	*GnuK*	Glucokinase	−1.33	+2.92	−1.41
PA2322	*GnuT*	Gluconate permease	−2.57	+1.74	−1.11
PA2338	*MtlE*	Maltose/mannitol ABC binding protein	Absent	Absent	Absent
PA2339	*MtlF*	Maltose/mannitol ABC permease protein	Absent	Absent	Absent
PA2340	*MtlG*	Maltose/mannitol ABC permease protein	NSS	NSS	NSS
PA2341	*MtlK*	Maltose/mannitol ABC ATP-binding protein	NSS	NSS	NSS
PA2342	*MltD*	Mannitol dehydrogenase	Absent	Absent	Absent
PA2344	*Frk*	Fructokinase	NSS	NSS	NSS
PA3181	*Eda*	2-Keto-3-deoxy-6-phosphogluconate adolase	+1.86	+2.04	−1.15
PA3183	*Zwf*	Glucose-6-phosphate dehydrogenase	+1.86	+2.07	−1.15
PA3186	*OprB*	Outer membrane porin OprB precursor	−4.08	−1.94	−1.37
PA3187	*GltK*	Glucose ABC ATP-binding protein	−7.25	−3.31	+1.25
PA3188	*GltG*	Glucose ABC permease protein	−12.94	−5.95	+1.10
PA3189	*GltF*	Glucose ABC permease protein	−17.15	−5.21	+1.37
PA3190	*GltB*	Glucose ABC binding protein	−10.56	−4.00	+1.67
PA3194	*Edd*	6-Phosphogluconate dehydratase	NSS	NSS	NSS
PA3561	*FruK*	Fructose-1-phophate kinase	−1.40	+1.23	+1.07
PA3581	*GlpF*	Glycerol uptake facilitator protein	+1.14	+7.33	−2.37
PA3582	*GlpK*	Glycerol kinase	+1.13	+6.06	−1.96
PA3584	*GlpD*	Glycerol-3-phosphate dehydrogenase	+1.36	+13.08	−1.57
PA3753	*Fdp*	Fructose-1,6-diphosphate aldolase	NSS	NSS	NSS
PA4732	*Pgi*	Phosphoglucoisomerase	−1.26	−1.39	+1.12
PA4748	*Tpi*	Triphosphate isomerase	NSS	NSS	NSS
PA5235	*GlpT*	Glycerol transport protein	−2.24	+4.40	−3.19

NNS Not statistically significant; *Absent* gene was not detected on the microarray

sodium hypochlorite exposure correlates with the twofold or more decrease in the genes related to oxidative phosphorylation (see Tables 2 and 3). After sodium hypochlorite exposure, all the genes associated with Complex I (NADH hydrogenase genes) were uniformly downregulated by threefold. Similarly, Complex II, which comprises fumerate reductase and other genes, were again all downregulated (~2.5-fold). Complex III, which contains the genes responsible for the cytochrome bc1 complex, were downregulated from twofold to 18-fold. The cytochrome c oxidase genes of Complex IV were all downregulated from twofold to 22-fold. However, peracetic acid and hydrogen peroxide exposure did not result in the same degree of downregulation of these genes. The transcriptional downregulation of these genes for the sodium hypochlorite-exposed samples suggested that both oxidative phosphorylation and the electron transport chain were significantly and uniformly downregulated, perhaps resulting in minimal energy production by these principal metabolic pathways after exposure. This uniform downregulation after sodium hypochlorite exposure is consistent with prior studies that demonstrated a decrease in respiratory function and the loss of metabolic energy after HOCl exposure (Albrich and Hurst 1982).

Carbon metabolism

The ED pathway and EMP pathway analysis also suggested that genes encoding carbon substrate catabolization during oxidative phosphorylation were affected by the disinfectant treatments (see Table 4). This pathway analysis also expanded upon the "Carbon compound catabolism," "Central intermediary metabolism," "Energy metabolism," "Membrane protein," and "Transport of small molecules" functional classes. The genes encoding the proteins that actively transport hexose molecules (glucose, maltose, gluconate, 2-ketogluconate, fructose, and glycerol) into the cell are downregulated after sodium hypochlorite exposure (but not after peracetic acid or hydrogen peroxide exposure). The genes that encode the transport of glucose (PA3186 to PA3190) across the periplasmic and cytoplasmic membranes were highly downregulated after sodium hypochlorite

Table 5 Comparison of organic sulfur transport gene expression for sodium hypochlorite-, peracetic acid-, and hydrogen peroxide-exposed *P. aeruginosa*

Gene number	Gene name	Gene description	NaOCl	CH_3CO_3H	H_2O_2
PA0183	*atsA*	Arylsulfatase	+3.81	+1.02	+1.11
PA0184	*AtsC*	Probable ATP-binding component of ABC sulfate ester transporter	+10.25	+1.73	+1.56
PA0185	*AtsB*	Probable permease of ABC sulfate ester transporter	+2.57	1.00	+1.20
PA0186	*AtsR*	Probable binding protein component of ABC sulfate ester transporter	+3.15	+1.12	−5.08
PA2307		Probable permease of ABC putative sulfonate transporter	+5.01	+1.93	+1.67
PA2308		Probable ATP-binding component of ABC putative sulfonate transporter	+5.19	−1.18	−2.41
PA2309		Hypothetical protein	+7.51	+1.67	−1.48
PA2310	*AtsK*	Alpha-ketoglutarate-dependent dioxygenase	+17.67	−1.18	−2.93
PA3441	*SsuF*	Molybdopterin-binding protein	+21.52	+1.36	−3.21
PA3442	*SsuB*	Probable ATP-binding component of ABC alkanesulfonate transporter	+37.84	+1.88	−2.35
PA3443	*SsuC*	Probable permease of ABC alkanesulfonate transporter	+34.41	+1.84	+1.70
PA3444	*SsuD*	Alkanesulfonate monooxygenase	+107.60	+1.61	+2.10
PA3445	*SsuA*	Conserved hypothetical protein	+103.00	+3.02	+1.24
PA3446	*SsuE*	NAD(P)H-dependent FMN reductase	+37.69	−1.06	1.00
PA3447		Probable ATP-binding component of ABC putative sulfonate transporter	+8.34	−1.39	+1.21
PA3448		Probable permease of ABC putative sulfonate transporter	+3.72	−1.38	−1.04
PA3449		Conserved hypothetical protein	+9.14	−2.88	−1.25
PA3450	*LsfA*	Peroxidredoxin (antioxidant protein)	+8.26	−1.53	−1.28
PA3935	*TauD*	Taurine dioxygenase	+10.35	+1.14	+1.22
PA3936	*TauC*	Probable permease of taurine ABC taurine transporter	+14.44	+1.19	+1.04
PA3937	*TauB*	Probable ATP-binding component of ABC taurine transporter	+12.07	+1.24	−1.05
PA3938	*TauA*	Probable periplasmic taurine-binding protein precursor	+22.65	−1.04	−1.04

exposure, and prior studies have shown glucose uptake to be inhibited after low concentrations of HOCl exposure (Schraufstatter et al. 1990). The genes encoding the same enzymes for these metabolites seemed to be either unaffected, or certainly nonsystemically affected, by hydrogen peroxide exposure. Peracetic acid exposure, however, resulted in downregulation of the same glucose transporter genes but to a lesser extent than upon sodium hypochlorite exposure. Exposure to sodium hypochlorite and hydrogen peroxide did not result in a significant upregulation of the glycerol or glycerol-3-phosphate transporter genes, which corresponds to studies that show glyceraldehyde-3-phosphate dehydrogenase is inactivated by sodium hypochlorite or hydrogen peroxide exposure (Schraufstatter et al. 1990). Additionally, the genes encoding glycerol and possibly glycerol-3-phosphate (PA3581, PA5235) showed a strong upregulation after peracetic acid exposure. The genes that code for the enzyme that catabolize glycerol, glycerol kinase (*glpK*, PA3582), and that catabolize glycerol-3-phosphate, glycerol-3-phosphate dehydrogenase (*glpD*, PA3584) were also strongly upregulated after peracetic acid exposure. This suggests that glycerol and glycerol-3-phospate may be a preferred substrate for growth as a result of peracetic acid exposure. They might also indicate an unrelated response to a potential carbon source rather than peracetic acid as an oxidant disinfectant. Indeed, prior studies have shown that glycerol and glycerol-3-phosphate are utilized as substrates for *P. aeruginosa* and *Escherichia coli* growth and are regulated by the potent feedback inhibition of fructose-1,6-diphosphate (Lessie and Phibbs 1984; McCowen et al. 1986). Additionally, *glpK* and *glpD* expression and activity are known to occur only when glycerol is utilized as a substrate for growth (McCowen et al. 1981). Furthermore, the Mg^{2+} transporter genes, *mgtA* (PA4825), was upregulated sixfold and *mgtC* (PA4635) was upregulated fourfold and could very well account for the excess Mg^{2+} ion required for glycerol kinase (*glpk*, PA3582) activity (McCowen et al. 1986). Hence, we suspect the observed response to peracetic acid compromises these components due to its antimicrobial activity as well as its potential as a carbon source.

Sulfur transport

The sodium hypochlorite exposure resulted in a strong upregulation of genes responsible for organic sulfur transport and the associated genes that catabolize these compounds (see Table 5). The genes encoding the alkanesulfonate transport system (PA3441 to PA3446) contained the most highly upregulated genes after sodium hypochlorite exposure and were not drastically upregulated or downregulated after peracetic acid or hydrogen peroxide exposure. *P. aeruginosa* has been known to use *n*-

alkanesulfonates or taurine as sources of both carbon and organic sulfur (van der Ploeg et al. 1996). Typically the *ssuD* (PA3444) and *ssuE* (PA3446) genes are expressed during sulfate or cysteine starvation (Eichhorn et al. 1999). This may suggest the following: that the sulfur in these compounds was required due to sulfur starvation caused by the reaction of the HOCl with sulfhydryl groups, that the carbon compound skeletons of the *n*-alkanesulfonates were being used in lieu of the carbon compounds contained in the hexose transporters of the ED pathway, or that the neutrophilic amines and alpha-amino acids formed by catabolization of *n*-alkanesulfonates may guard the cell against oxidative stress and attack from the HOCl moiety.

The genes encoding enzymes responsible for the taurine transport system (PA3935 to PA3938) exhibited the second most highly upregulated genes after sodium hypochlorite exposure and again were not drastically upregulated or downregulated in the peracetic acid-exposed or hydrogen peroxide-exposed samples. The *tauD* (PA3935) gene had a tenfold increase and is required for the catabolization of taurine to sulfite but has previously been shown to only be expressed under conditions of sulfate starvation (Eichhorn et al. 1997; van der Ploeg et al. 1996). Prior studies have demonstrated that taurine alone can be used as a sole source of carbon and sulfur in *P. aeruginosa* (Shimamoto and Berk 1979). Taurine has also been demonstrated to scavenge HOCl by forming *N*-chlorotaurine, which has greater stability and less toxicity (Grisham et al. 1984), and the *N*-chorotaurine has been shown to degrade to sulphoacetaldehyde in response to oxidative stress (Cunningham et al. 1998). This may suggest the following: that the sulfur in the taurine was required due to sulfur starvation caused by the reaction of HOCl with sulfhydryl groups, that the aldehyde carbon compounds were being utilized instead of the carbon compounds contained in the hexose transporters of the ED pathway, or that the taurine scavenged the highly destructive HOCl moiety by providing a less damaging pathway to degrade this compound.

The genes encoding proteins responsible for sulfate ester transporters (PA0183 to PA0186, PA2307 to PA2310) also had an upregulation after sodium hypochlorite exposure and again were not drastically upregulated or downregulated after peracetic acid or hydrogen peroxide exposure. The arylsulfatase gene, *atsA* (PA0183), was upregulated almost fourfold after sodium hypochlorite exposure and is associated with the sulfur starvation-induced proteins and has been used as a model system for the sulfate starvation response (Hummerjohann et al. 1998, 2000). Finally, the α-ketoglutarate-dependent dioxygenase gene, *atsK* (PA2310), was upregulated almost 18-fold after sodium hypochlorite exposure. The *atsK* enzyme catalyzes the oxidative conversion of the α-ketoglutarate cofactor into CO_2, succinate, and highly reactive ferryl (IV) species and is known to have a 38% amino acid homology to *tauD* (Muller et al. 2005). As these genes were strongly upregulated after sodium hypochlorite exposure and not changed after hydrogen peroxide or peracetic acid exposure, the *atsA* and *atsK* encoded enzymes may have directly reacted with the HOCl or performed side reactions to help mediate the oxidative stress caused by HOCl exposure.

Protective genes

Genes encoding several cellular protective mechanisms were highly upregulated after hydrogen peroxide exposure (see Supplementary Table 1). The catalase gene, *katA* (PA4236), did not show a significant upregulation for all three treatments which supports a prior suggestion that *katA* serves, instead, as a housekeeping catalase gene (Brown et al. 1995). However, the catalase gene, *katB* (PA4613), was upregulated sevenfold in the sodium hypochlorite-exposed samples and fourfold in the peracetic acid-exposed and hydrogen peroxide-exposed samples. This result corroborates the necessity of *katB* expression for optimal hydrogen peroxide resistance (Brown et al. 1995). The super oxide dismutase enzymes *sodB* (PA4366) and *sodM* (PA4468) did not show a significant upregulation or downregulation in all three treatments. However, the transcript raw signal intensities of *sodM* were among the highest for all of the treatments as well as the control samples (3,524 for sodium hypochlorite; 1,276 for peracetic acid; 1,834 for hydrogen peroxide; and 1923 for the control), thus suggesting that *sodB* gene expression may be considerable during standard aerobic growth conditions (Hassett et al. 1995). Several DNA repair-related genes were highly induced after hydrogen peroxide-exposure but were minimally upregulated after sodium hypochlorite and peracetic acid exposure. The DNA repair gene, *recN* (PA4763), was the exception as it was upregulated after all three treatments, although it was upregulated much more so after hydrogen peroxide exposure. These results again correspond well with prior studies establishing DNA damage with hydrogen peroxide exposure (Carlsson and Carpenter 1980; Demple and Halbrook 1983).

Iron regulation

Our previous studies confirmed that Fur-regulated genes were repressed after hydrogen peroxide exposure (Chang et al. 2005a; Palma et al. 2003). Comparison of three treatments (see Supplementary Table 2) to these iron-related genes revealed that exposure to sodium hypochlorite and peracetic acid also resulted in many of these same repressions in the following genes: ferri-siderophore or iron-chelating compound receptor genes, *fpvA* (PA2398) and *fptA* (PA4221); siderophore biosynthesis genes, *pchF*

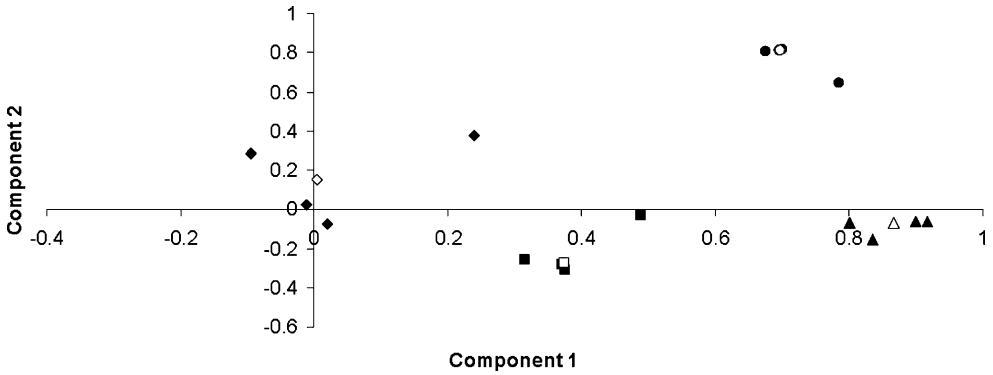

Fig. 3 PCA based on conditions for all samples. A scatter plot of all samples based on PCA component 1 (*x*-axis) versus PCA component 2 (*y*-axis). The *diamond-shaped points* (♦) are the control samples, the *square-shaped points* (■) are the hydrogen peroxide-exposed samples, the *circle-shaped points* (●) are the peracetic acid-exposed samples, and the *triangle-shaped points* (▲) are the sodium hypochlorite-exposed samples. The *outlined diamond* (◊) is the median of the control samples, the *outlined square* (□) is the median of the hydrogen peroxide-exposed samples, the *outlined circle* (○) is the median of the peracetic acid-exposed samples, and the *outlined triangle* (△) is the median of the sodium hypochlorite samples-exposed samples

(PA4225) and *pchE* (PA4226); pyochelin biosynthesis genes, pchDCBA (PA4228 to PA4231); siderophore or pyoverdin system-related genes (PA2403 to PA2410 and PA4156). Interestingly, the hydrogen peroxide responses are much higher than the strikingly similar sodium hypochlorite and peracetic acid responses to these iron regulation-related genes.

Pyocins

Perhaps the most striking result of our study was that the F-, R- and S-type pyocins (bacteriocins) of *P. aeruginosa* that were strongly upregulated after hydrogen peroxide exposure were downregulated after peracetic acid and sodium hypochlorite exposure (see Supplementary Table 3). These 41 genes were uniformly upregulated after hydrogen peroxide exposure and 37 of these bacteriocins were uniformly downregulated after sodium hypochlorite or peracetic acid exposure. Pyocins are readily induced by mutagenic agents (i.e., UV radiation and mitomycin C) and generally kill cells by degrading DNA and inhibiting lipid synthesis (Michel-Briand and Baysse 2002). Pyocin gene expression is regulated by the enzymes for *recA* (PA3617), *prtR* (PA0610), and *prtN* (PA0611); specifically, UV-activated RecA protein cleaves the repressor protein, PrtR, allowing the *prtN* gene to be expressed. The PrtN protein then facilitates the expression of various pyocin genes (Matsui et al. 1993). Thus, the upregulation of the *recA* gene combined with the possible RecA activation by hydroxyl radicals (instead of UV irradiation) may have caused the pyocin gene expression for the hydrogen peroxide-exposed samples. This is quite plausible, as the RecA protein has been shown to cleave single-stranded DNA with the aid of the hydroxyl radical (Akaboshi and Howard-Flanders 1990). However, the lack of *recA* expression after sodium hypochlorite and peracetic acid exposure may have prevented pyocin gene expression as well as the lack of an activator for the RecA protein.

Principal component analysis

The PCA on conditions analysis shows the closeness of all the samples to one another (see Fig. 3). Each set of samples for the three treatments and the control samples are grouped into four small clusters in the 3-D space. The closeness of the clusters was representative of the precision of the biological replicates for each sample set. The control samples groups predominantly around the origin as all samples were normalized to the control samples. Clearly, all three treatments did not function the same way as all the treatment samples do not appear in the same spatial areas compared to the control samples, which suggests that these three oxidative stressors did not affect the cells the same way.

In summary, the well-documented unparalleled effectiveness of sodium hypochlorite as a disinfectant coincides with a plethora of statistically significant genome-wide changes in gene transcription after sodium hypochlorite exposure, far more than in response to other oxidants peracetic acid or hydrogen peroxide. The genome-wide effects on hydrogen peroxide, particularly the upregulation of DNA repair genes, support previous studies that target DNA effects as the mechanism of action (McDonnell and Russell 1999). The mechanisms of action of sodium hypochlorite and peracetic acid appear to be related to membrane proteins. The increased transcription of genes associated with organic sulfur transport and decrease in genes associated with hexose compound transport across the cell

envelope supports prior reports of sodium hypochlorite's interaction with protein sulfhydryl groups and the down-regulation of respiratory systems associated with metabolic energy loss (Barrette et al. 1987). The resulting global transcription profile comparison can help identify commonly activated genes between these oxidative disinfectants. Furthermore, this information will help us better understand the mechanisms by which oxidative disinfectants kill bacteria.

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