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GENOMICS AND PROTEOMICS

Toxicogenomic analysis of sodium hypochlorite antimicrobial mechanisms in *Pseudomonas aeruginosa*

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Abstract Sodium hypochlorite (bleach) is routinely used in hospitals and health care facilities for surface sterilization; however, the mechanism of action by which this disinfectant kills and the extent to which *Pseudomonas aeruginosa* is resistant to sodium hypochlorite have not been elucidated. Consequently, nosocomial infections from *P. aeruginosa* result in considerable casualties and economic hardship. We report the genome-wide transcriptome response of *P. aeruginosa* to sodium hypochlorite-induced oxidative stress via the use of DNA microarrays. In addition to a general oxidative stress response, our data revealed a downregulation of virtually all genes related to oxidative phosphorylation and electron transport and an upregulation of many organic sulfur transport and metabolism genes.

Keywords Antimicrobial · Toxicogenomic · Microarray

Introduction

Oxidative antimicrobials (hydrogen peroxide, sodium hypochlorite, etc.) have commonly been used to eliminate pathogenic species such as *Pseudomonas aeruginosa* (Kitis 2004; Spoering and Lewis 2001). These compounds typically work by forming reactive oxygen species (ROS), which cause DNA and lipid damage (Miller and Britigan 1997). Bacteria have well-documented defense responses to chemically induced ROS (Chang et al. 2005a,b; Ochsner et al. 2000; Palma et al. 2004; Salunkhe et al. 2005; Zheng et al. 2001). However, bleach is uncommonly effective among oxidizers, suggesting more lethal combination of effects. The precise mechanisms by which sodium hypochlorite impacts and ultimately kills *P. aeruginosa* have not yet been elucidated (Rutala 1996). Given its superior antimicrobial activity and prevalence in many commercial disinfectants and detergents, this is surprising. We examined the transcriptional response of *P. aeruginosa* to sublethal levels of sodium hypochlorite to gain a more comprehensive understanding of the physiological response to sodium hypochlorite. *P. 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 and can result in debilitating and lethal illnesses (Tummler and Kiewitz 1999). *P. aeruginosa* is well suited to survive in a wide variety of environments: water, soil, and animals, and is prevalent in common surroundings (Campa et al. 1993). Infections caused by *P. aeruginosa* are typically difficult to treat due to the prominent resistance to antibiotics (Hancock 1998). In addition to confirming upregulation of oxidative stress response genes, this study revealed significant and coincident regulation of oxidative phosphorylation and organic sulfur metabolism genes. We interpreted this response as a potentially more lethal interplay between electron transport, sulfur metabolism, and oxidative stress.

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Materials and methods

Bacterial strains and growth conditions

P. aeruginosa PA01 overnight cultures were grown from freezer stocks in 100 ml aliquots (500 ml shaker flasks) of Luria–Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter) at 37°C, with shaking at 250 rpm for 17 h. The overnight cultures were diluted 1:100 in 25 ml aliquots (250 ml shaker flasks) of prewarmed LB broth and incubated at 37°C, with shaking at 250 rpm until the optical density at 600 nm (OD_{600}) reached mid logarithmic phase (OD_{600}~0.8). A final dilution of 1:10 prewarmed LB broth in 25 ml aliquots (250 ml shaker flasks) was performed, and the cultures were incubated at 37°C, with shaking at 250 rpm until the optical density at 600 nm (OD_{600}) reached mid logarithmic phase (OD_{600}~0.8). Various concentrations of sodium hypochlorite (Aldrich Chemical) were added immediately after OD_{600} reached 0.8. Cell growth was monitored by measuring OD_{600} with a Lambda 25 spectrophotometer (Perkin Elmer).

RNA isolation

For microarray analysis, RNA was isolated 20 min after the addition of sodium hypochlorite using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNAprotect Bacteria Reagent (Qiagen) was also added before the isolation for stabilization. Cells were incubated in the RNAprotect Bacteria Reagent for 5 min, harvested by centrifugation (>8,000×*g*), and then incubated in TE buffer with 1 mg/ml of lysozyme (Roche Applied Science). Finally, samples were eluted with 50 μl of nuclease-free water (Ambion). RNA quality, purity, and integrity were determined using both a Lambda 25 spectrophotometer (Perkin Elmer) and an RNA 6000 Nano LabChips with an Agilent 2100 Bioanalyzer (Agilent Technologies).

cDNA synthesis and labeling

cDNA was synthesized from 12 μg of RNA with random primers and SuperScript II (both from Invitrogen) according to the protocol for the Affymetrix *P. aeruginosa* GeneChip array (Affymetrix). Control transcripts from *Bacillus subtilis* genes *dap*, *thr*, *phe*, and *lys* (Affymetrix) were spiked into RNA mixtures to monitor labeling, hybridization, and staining efficiency. The cDNA purified with a QIAquick PCR purification kit (Qiagen) was then fragmented at 37°C for 10 min by the addition of DNase I (0.06 U/μg of cDNA) (Roche Applied Science) in One Phor-All buffer (Invitrogen). The Enzo BioArray Terminal Labeling Kit with Biotin-ddUTP (Enzo Life Sciences) was utilized to label 3′ termini of fragmented cDNA.

Hybridization and scanning

Hybridization cocktail was prepared with fragmented and labeled cDNA and B2 control oligonucleotide (Affymetrix). The cocktail was hybridized onto *P. aeruginosa* GeneChip arrays (Affymetrix) at 50°C for 16 h. The arrays were washed and stained with ImmunoPure streptavidin (Pierce Biotechnology), anti-streptavidin goat antibody (Vector Laboratories, Burlingame, CA, USA), and R-phycoerythrin streptavidin (Molecular Probes) using a GeneChip Fluidics Station 450 (Affymetrix). Finally, the arrays were scanned with the GeneChip Scanner 3000 (Affymetrix).

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: α_1= 0.04, α_2=0.06, and τ=0.015; target signal was scaled to 150. Genes that received "absent" calls from 50% or more of the replicates in GeneSpring were not used for the analysis. Finally, gene expression changes with statistical significance were identified by an upper one-tailed *t* test (*p* cutoff value, 0.05). "Fold change" was calculated as the ratio between the signal averages of four untreated and four treated cultures. That is, results are from biological quadruplicate experiments. Genes with a twofold or more induction or repression were used in this analysis.

Real-time PCR analysis for microarray validation

mRNA transcript level changes obtained via the microarray analysis were evaluated by quantitative real-time PCR (e.g., *katB*, *ohr*, *recN*, and PA5530 genes). These genes were selected because they showed a range of mRNA level increases (three- to 12-fold). PA0576 (*rpoD*) was also used as a control gene as its expression level is steady (Savli et al. 2003). Genes and primer sequences used for the real-time PCR analysis were designed using Beacon Designer v. 3.0 (Premier Biosoft International) and are found in Table 1. The real-time PCR was performed by using the iCycler IQ Real-Time PCR Detection System with iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (BioRad Laboratories). The values obtained by real-time PCR have a good correspondence to those obtained by the microarray (Table 1).

Results

P. aeruginosa transcriptome changes in response to oxidative stress

A sublethal exposure of 4.4 mM (0.03%) sodium hypochlorite for 20 min showed inhibition of growth (~50%) without noticeable cellular death and enabled mRNA analysis (see Fig. 1). Of the 5,570 genes in the *P. aeruginosa* genome, 2,435 genes showed statistical significance based on a *t* test. We found that mRNA levels of 1,101 (of 5,570) *P. aeruginosa* genes were significantly altered in response to bleach by twofold or more (471 upregulated, 630 downregulated).

Functional classifications analysis

Functional classifications of the responding genes are provided in Fig. 2 (the hypothetical, unclassified, and unknown were omitted). Functional classes are taken from *P. aeruginosa* Community Annotation Project, http://v2.pseudomonas.com/ (Winsor et al. 2005).

Metabolic pathway analysis

P. aeruginosa pathways from the Kyoto Encyclopaedia of Genes and Genomes (Ogata et al. 1999) were downloaded and imported into the GeneSpring GX genome software and visually inspected for changes based on the 1,101 genes from the *t*-test analysis. These metabolic pathways were compiled in tables to organize the data based on metabolic pathways. Striking features were revealed by inspection. First, the oxidative phosphorylation pathway genes of all five complexes (Ogata et al. 1999) were compiled and organized in Fig. 3 and Table 2. All genes were significantly downregulated. Second, the Enteroff–Doudoroff (ED) pathway and Embden–Meyerhof–Parnas (EMP) pathway were organized in Table 3 (Roehl et al. 1983; Roehl and Phibbs 1982); a significant number of genes were downregulated. Third, ATP-binding cassettes (ABC) for organic sulfur transport-related genes were compiled and organized in Table 4 based along with glucose transport genes (Ogata et al. 1999). Sulfur transport genes were nearly uniformly upregulated at the same time that glucose transport genes were downregulated.

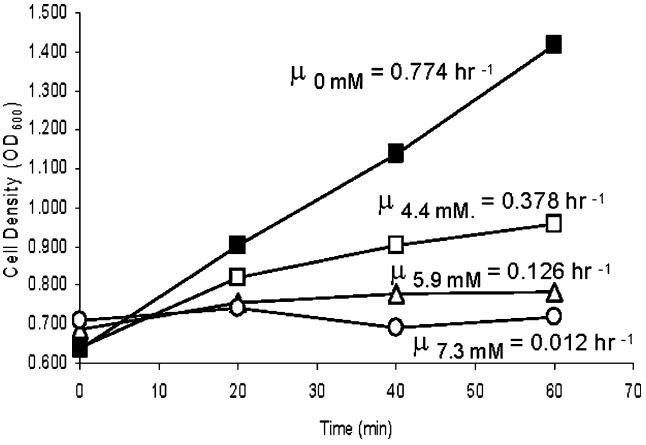

Fig. 1 Growth inhibition of *P. aeruginosa* exposed to sodium hypochlorite. Growth data and growth rates for 1 h postexposure to sodium hypochlorite are shown. The sodium hypochlorite concentrations were as follows: 0 mM, control (*filled square*), 4.4 mM (*empty square*), 5.9 mM (*triangle*), and 7.3 mM (*circle*). The growth rates (μ) of each sodium hypochlorite concentration are shown next to each respective sample

Discussions

As expected, sodium hypochlorite treatment triggered the expression of genes involved in antioxidant adaptation and protection process, which are conserved among Eubacteria. Sodium hypochlorite has been proposed and shown to elicit similar responses to hydrogen peroxide by generating

Table 1 Transcript level comparison of *P. aeruginosa* genes between real-time PCR analysis and microarray analysis

Gene	mRNA level change with real-time PCR	mRNA level change with microarray	Sense primer sequence	Antisense primer sequence
PA4613 (*katB*)	3.66 (±0.25)	+7.50	5′-GAGCAGAACTTCAAGCAGAC-3′	5′-CTCTCGTCGTCGGTGATC-3′
PA2850 (*ohr*)	23.43 (±1.89)	+21.50	5′-GAGGTCGAACTGCACATC-3′	5′-GGGTAGCGTTGGAGTAGG-3′
PA4763 (*recN*)	1.28 (±0.09)	+2.42	5′-GGAGCAGGAGCAGAAGAC-3′	5′-GTTGAGGCTGGCATTGAG-3′
PA5530	7.20 (±1.68)	+14.95	5′-AAGAAGGAAGAGCCGAAGG-3′	5′-ATGTAGGTGGTGTAGGTGTAG-3′
PA0576 (*rpoD*)[a]	1.00	1.00	5′-CGTCCTCAGCGGCTATATCG-3′	5′-TTCTTCTTCCTCGTCGTCCTTC-3′

[a] PA0576 (*rpoD*) was used as the house-keeping gene.

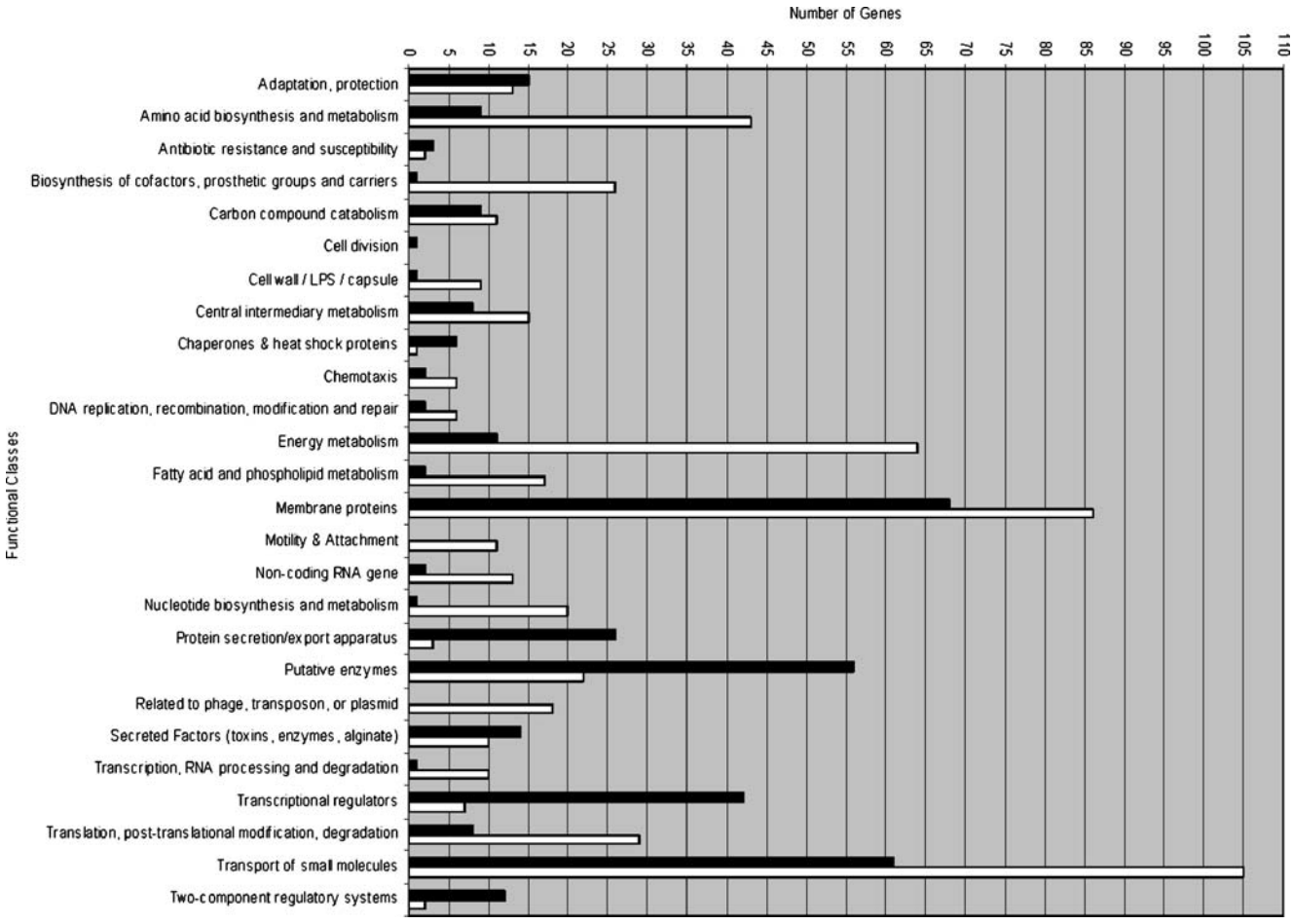

Fig. 2 Function classification of number of genes with statistically significant increase and decrease in mRNA level. Functional classification of genes with increased (*filled square*) and decreased (*empty square*) mRNA level changes of twofold or more after 20 min exposure to 4.4 mM sodium hypochlorite

superoxide anions (oxygen singlets) and hydroxyl radicals, which were presumed to account for the major bactericidal activity (Albrich and Hurst 1982; Candeias et al. 1993; Dukan and Touati 1996; Imlay and Linn 1986; Khan and Kasha 1994a,b). Hence, it has been speculated that sodium hypochlorite functions by similar mechanisms as other oxidizing agents (Miller and Britigan 1997). Correspondingly, oxidant defense system genes using catalase (*kat*), alkyl hydroperoxide reductase (*ahp*), and glutathione peroxidase/reductase (Block 2001; Dukan and Touati 1996) were all upregulated. Catalase gene *katB*, PA4613, was strongly induced (sevenfold), although *katA* (PA4236) did not show a significant fold increase (less than twofold), which is consistent with other reports (Brown et al. 1995; Hassett et al. 2000; Ochsner et al. 2000). These largely confirmatory results demonstrate that the experimental design (e.g., treatment level of sodium hypochlorite and sample time) was appropriate for determining the global physiological response of *P. aeruginosa* to hypochlorite.

Exposure to sodium hypochlorite, however, repressed a variety of genes involved in primary metabolic processes including fatty acid biosynthesis and energy metabolism. Most notably, oxidative phosphorylation and electron transport systems were among the most heavily downregulated (Fig. 2 and Table 2). The components of oxidative phosphorylation are organized into interconnecting protein assemblies (Complexes I–IV). NADH dehydrogenase genes in Complex I were uniformly downregulated by threefold. Fumarate reductase and all associated genes in Complex II were similarly all downregulated (~2.5-fold). The genes in the cytochrome bc1 complex (Complex III) were downregulated from two- to 18-fold. Cytochrome *c* oxidase and associated Complex IV genes were all downregulated two- to 22-fold. Also, all elements of respiratory anoxic redox control (*arcDABC* operon) system were downregulated threefold (Table 1), which is consistent with the observed inhibition of autophosphorylation by quinone electron carriers under oxidative stress (Georgellis et al. 2001). The transcriptional downregulation of these genes suggests that both oxidative phosphorylation and the electron transport chain were significantly and uniformly impaired, perhaps resulting in minimal energy production

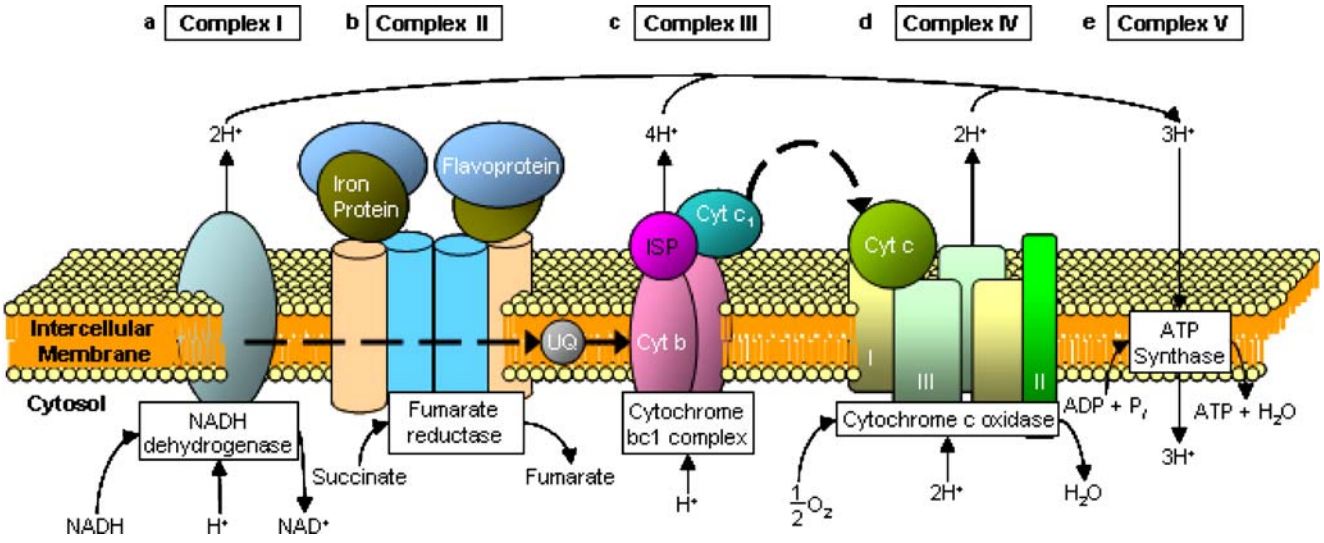

Fig. 3 Genes associated with oxidative phosphorylation and electron transport were nearly uniformly downregulated (33 downregulated genes contained in the four complexes). **a** NADH dehydrogenase subunits (Complex I) were nearly uniformly downregulated by twofold. **b** Fumarate reductase and succinate dehydrogenase and all four subunits (Complex II) were downregulated by twofold. **c** Complex III contains the most highly downregulated genes in the transcriptome, the genes encoding iron–sulfur proteins. **d** Cytochrome *c* oxidases (Complex IV) and associated genes were downregulated between two- and 21-fold. **e** Genes responsible for ATP synthase (Complex V) were downregulated by approximately twofold (data not shown)

by these principal metabolic pathways. This uniform downregulation is consistent with prior studies that demonstrated a decrease in respiratory function after hypochlorous acid exposure (Albrich and Hurst 1982; Albrich et al. 1981; Dukan and Touati 1996).

The ED pathway and EMP pathway analysis also suggested that genes encoding carbon substrate catabolization during oxidative phosphorylation were affected by sodium hypochlorite exposure (see Table 3). 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 for sodium-hypochlorite-exposed samples. The genes that encode the transport of glucose (PA3186 to PA3190) across the periplasmic and cytoplasmic membranes were highly downregulated, and prior studies have shown glucose uptake to be inhibited after low concentrations of hypochlorous acid exposure (Schraufstatter et al. 1990). The OprB porin (PA3186) is described more appropriately as a carbohydrate-selective porin because it facilitates the diffusion of a wide range of carbohydrates across the outer membrane in addition to glucose (Wylie and Worobec 1995). The sodium-hypochlorite-exposed samples did not show a significant upregulation of the glycerol or glycerol-3-phosphate transporter genes, which corresponds to studies that show that glyceraldehyde-3-phosphate dehydrogenase is inactivated by oxidative stressors (Schraufstatter et al. 1990).

It is interesting to note that our data showed that genes associated with the active transport of the following organic sulfur compounds were highly upregulated: taurine, alkanesulfonate, sulfonate, sulfate ester, and sulfate (see Table 4). Biological substrates containing sulfhydryl groups (i.e., iron–sulfur proteins, β-carotenes, porphyrins, heme proteins, nucleotides, and enzymes containing essential cysteine molecules) are abundant in available electrons (e.g., for reaction, sharing, etc.) and are considered extremely reactive with strong oxidizers such as the hypochlorous acid formed from sodium hypochlorite (Albrich et al. 1981; Harrison et al. 1978b). The genes encoding the alkanesulfonate transport system (PA3441 to PA3446) contained the most highly upregulated genes after sodium hypochlorite exposure. *P. aeruginosa* has been known to use *n*-alkanesulfonates or taurine as sources of both carbon and organic sulfur. 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 neutrophil amines and alpha-amino acids formed by catabolization of *n*-alkanesulfonates may guard the cell against oxidative stress and attack from HOCl.

The genes encoding enzymes responsible for the taurine transport system (PA3935 to PA3938) exhibited the second most highly upregulated genes after sodium hypochlorite

Table 2 *P. aeruginosa* genes related to oxidative phosphorylation and electron transport

Gene (name)	Fold change	*p* value	Description
Complex I, NADH dehydrogenase I			
PA2637 (*nuoA*)	−2.21	0.004	NADH dehydrogenase I chain A
PA2638 (*nuoB*)	−3.08	0.004	NADH dehydrogenase I chain B
PA2639 (*nuoD*)	−2.92	0.004	NADH dehydrogenase I chain C, D
PA2640 (*nuoE*)	−2.70	0.003	NADH dehydrogenase I chain E
PA2641 (*nuoF*)	−2.72	0.004	NADH dehydrogenase I chain F
PA2642 (*nuoG*)	−3.07	0.003	NADH dehydrogenase I chain G
PA2643 (*nuoH*)	−3.08	0.003	NADH dehydrogenase I chain H
PA2644 (*nuoI*)	−3.48	0.003	NADH dehydrogenase I chain I
PA2645 (*nuoJ*)	−3.48	0.002	NADH dehydrogenase I chain J
PA2646 (*nuoK*)	−3.49	0.003	NADH dehydrogenase I chain K
PA2647 (*nuoL*)	−3.86	0.003	NADH dehydrogenase I chain L
PA2648 (*nuoM*)	−3.62	0.003	NADH dehydrogenase I chain M
PA2649 (*nuoN*)	−3.75	0.002	NADH dehydrogenase I chain N
Complex II, fumarate reductase			
PA1581 (*sdhC*)	−2.56	0.003	Fumarate reductase C subunit
PA1582 (*sdhD*)	−2.40	0.004	Fumarate reductase D subunit
PA1583 (*sdhA*)	−2.44	0.002	Fumarate reductase A subunit
PA1584 (*sdhB*)	−2.44	0.004	Fumarate reductase B subunit
Complex III, cytochrome bc1 complex			
PA4131	−17.79	0.002	Probable iron–sulfur protein
PA4429	−3.46	0.001	Probable cytochrome c1 precursor
PA4430	−3.44	0.004	Probable cytochrome b
PA4431	−2.28	0.002	Probable iron–sulfur protein
Complex IV, cytochrome *c* oxidase			
PA1317 (*cyoA*)	−5.59	0.002	Ubiquinol oxidase subunit II
PA1318 (*cyoB*)	−3.57	0.003	Ubiquinol oxidase subunit I
PA1319 (*cyoC*)	−4.12	0.003	Ubiquinol oxidase subunit III
PA1320 (*cyoD*)	−5.29	0.021	Ubiquinol oxidase subunit IV
PA1321 (*cyoE*)	−2.53	0.008	Ubiquinol oxidase protein CyoE
PA1552	−4.85	0.003	Probable cytochrome *c*
PA1553	−4.63	0.002	Cytochrome *c* oxidase subunit
PA1554	−3.85	0.002	Subunit (cbb3-type)
PA1555	−12.58	0.003	Probable cytochrome *c*
PA1556	−14.47	0.003	Cytochrome *c* oxidase subunit
PA1557	−21.65	0.003	Cytochrome oxidase subunit
PA4133	−5.05	0.003	Subunit (cbb3-type)

exposure. 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; Shimamoto and Berk 1980a,b). Taurine has also been demonstrated to scavenge HOCl by forming *N*-chlorotaurine which has greater stability and less toxicity (Grisham et al. 1984; Harrison et al. 1978a; Weiss et al. 1982), and the *N*-chlorotaurine has been shown to degrade to sulfoacetaldehyde 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 the 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 stabilized the highly destructive HOCl providing a less damaging pathway to degrade this compound. It is interesting that the genes encoding taurine-depleting enzyme, and gamma-glutamyl-transpeptidase enzyme, and PA1338 (*ggt*), which yields 5-glutamyl-taurine, were downregulated sixfold, suggesting that taurine may be channeled into sulfite-producing pathways.

The genes encoding proteins responsible for sulfate ester transporters (PA0183 to PA0186, and PA2307 to PA2310) also had an upregulation after sodium hypochlorite exposure and again were not drastically upregulated or down-

Table 3 Enteroff–Doudoroff (ED) pathway and Embden–Meyerhof–Parnas (EMP) pathway gene expression for sodium hypochlorite-exposed *P. aeruginosa*

Gene (name)	Fold change	*p* value	Description
PA0337 (*pts*)	NSS[b]	NSS[b]	Phosphotransferase system
PA0555 (*fda*)	NSS[b]	NSS[b]	Fructose-1,6-diphosphate aldolase
PA2262 (*kguT*)	NSS[b]	NSS[b]	Probable 2-ketogluconate transporter
PA2265 (*gad*)	−1.88	0.004	Gluconate dehydrogenase
PA2290 (*gcd*)	−2.95	0.002	Glucose dehydrogenase
PA2321 (*gnuK*)	NSS[b]	NSS[b]	Glucokinase
PA2322 (*gnuT*)	−2.57	0.010	Gluconate permease
PA2338 (*mtlE*)	Absent[a]	Absent[a]	Maltose/mannitol ABC-binding protein
PA2339 (*mtlF*)	Absent[a]	Absent[a]	Maltose/mannitol ABC permease protein
PA2340 (*mtlG*)	NSS[b]	NSS[b]	Maltose/mannitol ABC permease protein
PA2341 (*mtlK*)	NSS[b]	NSS[b]	Maltose/mannitol ABC ATP-binding protein
PA2342 (*mltD*)	Absent[a]	Absent[a]	Mannitol dehydrogenase
PA2344 (*frk*)	NSS[b]	NSS[b]	Fructokinase
PA3181 (*eda*)	+1.86	0.005	2-Keto-3-deoxy-6-phosphogluconate aldolase
PA3183 (*zwf*)	+1.86	0.004	Glucose-6-phosphate dehydrogenase
PA3186 (*oprB*)	−4.08	0.002	Outer membrane porin OprB precursor
PA3187 (*gltK*)	−7.25	0.003	Glucose ABC ATP-binding protein
PA3188 (*gltG*)	−12.94	0.005	Glucose ABC permease protein
PA3189 (*gltF*)	−17.15	0.007	Glucose ABC permease protein
PA3190 (*gltB*)	−10.56	0.003	Glucose ABC-binding protein
PA3194 (*edd*)	NSS[b]	NSS[b]	6-Phosphogluconate dehydratase
PA3561 (*fruK*)	−1.40	0.037	Fructose-1-phosphate kinase
PA3581 (*glpF*)	NSS[b]	NSS[b]	Glycerol uptake facilitator protein
PA3582 (*glpK*)	NSS[b]	NSS[b]	Glycerol kinase
PA3584 (*glpD*)	NSS[b]	NSS[b]	Glycerol-3-phosphate dehydrogenase
PA3753 (*fdp*)	NSS[b]	NSS[b]	Fructose-1,6-diphosphate aldolase
PA4732 (*pgi*)	NSS[b]	NSS[b]	Phosphoglucoisomerase
PA4748 (*tpi*)	NSS[b]	NSS[b]	Triphosphate isomerase
PA5235 (*glpT*)	NSS[b]	NSS[b]	Glycerol transport protein

[a] Gene was not detected on the microarray.
[b] Not statistically significant according to *t* test

regulated in the peracetic-acid-exposed or hydrogen-peroxide-exposed samples (Chang et al. 2005a,b). The arylsulfatase gene, *atsA* (PA0183), was upregulated almost fourfold in the sodium-hypochlorite-exposed samples 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; Hummerjohann et al. 2000; Quadroni et al. 1999). The α-ketoglutarate-dependent dioxygenase gene, *atsK* (PA2310), was upregulated almost 18-fold in the sodium-hypochlorite-exposed samples. The AtsK enzyme catalyzes the oxidative conversion of 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* (Kahnert and Kertesz 2000; Muller et al. 2005). Because these genes were strongly upregulated in the sodium-hypochlorite-exposed samples, 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 hypochlorous acid exposure.

PA2025 (*gor*) and PA2826, which were both upregulated sevenfold after the sodium hypochlorite treatment, are reportedly related to glutathione reductase and glutathione peroxidase, respectively. Additionally, PA1773, leading to a putative glutathione peroxidase, potentially reduces organic hydroperoxides to the corresponding alcohols and was not induced significantly (less than twofold). Glutathione peroxidase removes hydrogen peroxide with the aid of glutathione. Moreover, glutathione reductase recycles glutathione for further oxidative removal. Thus, it is presumed that PA2025 and PA2826 played those roles for the detoxification of oxidative products produced in the presence of sodium hypochlorite. It is interesting to note that the neighboring genes, PA2825 (probable transcriptional regulator) and PA2827 (probable methionine-*S*-oxide reductase), were also among the genes that were most

Table 4 *P. aeruginosa* genes related to the transport of small molecules

Gene (name)	Fold change	*p* value	Description
Glucose			
PA3186	−4.08	0.003	Probable ATP-binding component of ABC transporter
PA3187	−7.25	0.007	Probable permease of ABC sugar transporter
PA3188	−12.94	0.005	Probable permease of ABC sugar transporter
PA3189	−17.15	0.003	Probable binding protein component of ABC sugar transporter
PA3190 (*oprB*)	−10.56	0.002	Outer membrane porin OprB precursor
Iron (III)			
PA4687 (*hitA*)	−6.76	0.002	Ferric iron-binding periplasmic protein
PA4688 (*hitB*)	−5.52	0.002	Iron (III) transport system permease
Sulfonate (putative)			
PA3447	+8.34	0.011	Probable ATP-binding component of ABC transporter
PA3448	+3.72	0.005	Probable permease of ABC sugar transporter
PA3449	+9.14	<0.001	Probable binding protein component of ABC sugar transporter
PA3450 (*lsfA*)	+8.26	<0.001	Probable antioxidant protein
Sulfonate (putative)			
PA3441 (*ssuF*)	+21.52	<0.001	Probable molybdopterin-binding protein
PA3442	+37.84	<0.001	Probable ATP-binding component of ABC transporter
PA3443	+34.41	<0.001	Probable permease of ABC transporter
PA3444 (*ssuD*)	+107.60	<0.001	Probable alkane sulfonate monooxygenase
PA3445	+103.00	<0.001	Conserved hypothetical protein
PA3446 (*ssuE*)	+37.69	<0.001	Probable NAD(P)H-dependent FMN reductase
Taurine			
PA3935 (*tauD*)	+10.35	<0.001	Taurine dioxygenase
PA3936	+14.44	<0.001	Probable permease of ABC taurine transporter
PA3937	+12.07	<0.001	Probable ATP-binding component of ABC taurine transporter
PA3938	+22.65	<0.001	Probable periplasmic taurine-binding protein precursor
Sulfate ester			
PA0183 (*atsA*)	+3.81	<0.001	Arylsulfatase
PA0184	+10.25	<0.001	Probable ATP-binding component of ABC transporter
PA0185	+2.57	<0.001	Probable permease of ABC transporter
PA0186	+3.15	<0.001	Probable binding protein component of ABC transporter
Sulfonate (putative)			
PA2307	+5.01	0.00407	Arylsulfatase
PA2308	+5.19	0.000488	Probable ATP-binding component of ABC transporter
PA2309	+7.51	1.21×10^{-6}	Probable permease of ABC transporter
PA2310 (*atsK*)	+17.67	1.85×10^{-7}	Probable binding protein component of ABC transporter

strongly induced by sodium hypochlorite (upregulated seven- and 23-fold, respectively).

This study also revealed that sodium hypochlorite induced several alkyl hydroperoxide reductase genes such as *ohr*, *ahpC*, and *ahpF*, which are essential for optimal resistance to oxidative stress compounds (Cussiol et al. 2003; Ochsner et al. 2000). We suspect that intracellular H_2O_2 generated from sodium hypochlorite exposure was most likely broken down by Ohr because the organic hydroperoxide resistance gene PA2850 (*ohr*) showed an increase of 22-fold. On the other hand, PA0139 (*ahpC*) did not show a statistical significant increase, and PA0140 (*ahpF*) was insignificantly upregulated (less than twofold). This result could be explained by the fact that *ahpC* is normally expressed at a high level during aerobic growth (Ochsner et al. 2000). Previous studies have shown that Ohr catalytic activity is indeed thiol dependent and may be related to the increases in sulfur metabolism (Cussiol et al. 2003). We believe that an intracellular accumulation of sulfur compounds, coincident with upregulation of their transport genes, provides additional substrates for peroxide destruction.

The present study represents the first genome-wide response of any microbe exposed to sodium hypochlorite. Owing to its pathogenicity and ubiquitous presence in clinical settings, *P. aeruginosa* was selected as a model organism. We have attempted to discover the bactericidal mechanisms by which sodium hypochlorite functions. The results in this study support the following conclusions: (1) sodium hypochlorite is an oxidant and initiates a stress response similar to that of other oxidants including hydrogen peroxide; (2) sodium hypochlorite severely represses genes involved in energy generation (glucose

transport, oxidative phosphorylation, and electron transport); (3) sodium hypochlorite induces genes responsible for oxidizing sulfur compounds to sulfites and sulfates, which is consistent with the hypothesis that organic sulfur compounds facilitate much needed energy production; and (4) sodium hypochlorite induces genes encoding peroxidases and antioxidants related to cellular protective processes that neutralize the direct effects of sodium hypochlorite. Several genes responsible for two-component control systems support these conclusions.

This gene expression profile can now be utilized more closely to examine the specific mechanisms by which bacterial cells that die develop resistance to sodium hypochlorite, as well as the modes of action by which other biocides affect *P. aeruginosa*. The net response of *P. aeruginosa* to sodium hypochlorite is an amalgamation of cellular functions that are coordinately affected resulting in efficient lethality. We suggest that the cells combat the oxidizing agent via the typical ROS response, but because of the coincident downregulation of oxidative phosphorylation, they are starved for energy. A net decrease in glucose uptake (depression of uptake genes) may coincide with uptake of organic sulfur compounds as an alternative carbon source. The release of reduced or partially oxidized sulfur upon degradation of organic sulfur substrates provides an alternative electron sink. While these are speculatory, additional studies are needed to fully address these issues. We suspect that the multigenic impact of sodium hypochlorite is a sufficient motivation to carry out this work; perhaps, coincident targeting of distantly related genes may offer advantages for next generation antimicrobials. Furthermore, given that biofilms are considered to be highly resistant to antimicrobial agents compared to planktonic cells, a detailed comparative examination of transcriptome profiles of biofilms vs planktonic cells upon exposure to sodium hypochlorite will be another interesting topic for future studies.

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References

Albrich JM, Hurst JK (1982) Oxidative inactivation of Escherichia coli by hypochlorous acid. Rates and differentiation of respiratory from other reaction sites. FEBS Lett 144:157–161

Albrich JM, McCarthy CA, Hurst JK (1981) Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc Natl Acad Sci U S A 78: 210–214

Block SS (2001) Disinfection, sterilization, and preservation. Williams & Wilkins, Philadelphia, PA

Brown SM, Howell ML, Vasil ML, Anderson AJ, Hassett DJ (1995) Cloning and characterization of the katB gene of Pseudomonas aeruginosa encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. J Bacteriol 177:6536–6544

Campa M, Bendinelli M, Friedman H (1993) *Pseudomonas aeruginosa* as an opportunistic pathogen. Plenum, New York

Candeias LP, Patel KB, Stratford MR, Wardman P (1993) Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. FEBS Lett 333:151–153

Chang W, Small DA, Toghrol F, Bentley WE (2005a) Microarray analysis of Pseudomonas aeruginosa reveals induction of pyocin genes in response to hydrogen peroxide. BMC Genomics 6:115

Chang W, Small DA, Toghrol F, Bentley WE (2005b) Microarray analysis of toxicogenomic effects of peracetic acid on Pseudomonas aeruginosa. Environ Sci Technol 39:5893–5899

Cunningham C, Tipton KF, Dixon HB (1998) Conversion of taurine into N-chlorotaurine (taurine chloramine) and sulphoacetaldehyde in response to oxidative stress. Biochem J 330(Pt 2):939–945

Cussiol JR, Alves SV, de Oliveira MA, Netto LE (2003) Organic hydroperoxide resistance gene encodes a thiol-dependent peroxidase. J Biol Chem 278:11570–11578

Dukan S, Touati D (1996) Hypochlorous acid stress in Escherichia coli: resistance, DNA damage, and comparison with hydrogen peroxide stress. J Bacteriol 178:6145–6150

Eichhorn E, van der Ploeg JR, Kertesz MA, Leisinger T (1997) Characterization of alpha-ketoglutarate-dependent taurine dioxygenase from *Escherichia coli*. J Biol Chem 272:23031–23036

Eichhorn E, van der Ploeg JR, Leisinger T (1999) Characterization of a two-component alkanesulfonate monooxygenase from *Escherichia coli*. J Biol Chem 274:26639–26646

Georgellis D, Kwon O, Lin EC (2001) Quinones as the redox signal for the arc two-component system of bacteria. Science 292:2314–2316

Grisham MB, Jefferson MM, Melton DF, Thomas EL (1984) Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. J Biol Chem 259:10404–10413

Hancock RE (1998) Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria. Clin Infect Dis 27(Suppl 1):S93–S99

Harrison JE, Watson BD, Schultz J (1978a) Myeloperoxidase and singlet oxygen: a reappraisal. FEBS Lett 92:327–332

Harrison JE, Watson BD, Schultz J (1978b) Myeloperoxidase and singlet oxygen: a reappraisal. FEBS Lett 92:327–332

Hassett DJ, Alsabbagh E, Parvatiyar K, Howell ML, Wilmott RW, Ochsner UA (2000) A protease-resistant catalase, KatA, released upon cell lysis during stationary phase is essential for aerobic survival of a Pseudomonas aeruginosa oxyR mutant at low cell densities. J Bacteriol 182:4557–4563

Hummerjohann J, Kuttel E, Quadroni M, Ragaller J, Leisinger T, Kertesz MA (1998) Regulation of the sulfate starvation response in *Pseudomonas aeruginosa*: role of cysteine biosynthetic intermediates. Microbiology 144(Pt 5):1375–1386

Hummerjohann J, Laudenbach S, Retey J, Leisinger T, Kertesz MA (2000) The sulfur-regulated arylsulfatase gene cluster of *Pseudomonas aeruginosa*, a new member of the cys regulon. J Bacteriol 182:2055–2058

Imlay JA, Linn S (1986) Bimodal pattern of killing of DNA-repair-defective or anoxically grown Escherichia coli by hydrogen peroxide. J Bacteriol 166:519–527

Kahnert A, Kertesz MA (2000) Characterization of a sulfur-regulated oxygenative alkylsulfatase from *Pseudomonas putida* S-313. J Biol Chem 275:31661–31667

Khan AU, Kasha M (1994a) Singlet molecular oxygen evolution upon simple acidification of aqueous hypochlorite: application to studies on the deleterious health effects of chlorinated drinking water. Proc Natl Acad Sci U S A 91:12362–12364

Khan AU, Kasha M (1994b) Singlet molecular oxygen in the Haber–Weiss reaction. Proc Natl Acad Sci U S A 91:12365–12367

Kitis M (2004) Disinfection of wastewater with peracetic acid: a review. Environ Int 30:47–55

Miller RA, Britigan BE (1997) Role of oxidants in microbial pathophysiology. Clin Microbiol Rev 10:1–18

Muller I, Stuckl C, Wakeley J, Kertesz M, Uson I (2005) Succinate complex crystal structures of the alpha-ketoglutarate-dependent dioxygenase AtsK: steric aspects of enzyme self-hydroxylation. J Biol Chem 280:5716–5723

Ochsner UA, Vasil ML, Alsabbagh E, Parvatiyar K, Hassett DJ (2000) Role of the Pseudomonas aeruginosa oxyR-recG operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of katB-ankB, ahpB, and ahpC-ahpF. J Bacteriol 182:4533–4544

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M (1999) KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 27:29–34

Palma M, DeLuca D, Worgall S, Quadri LE (2004) Transcriptome analysis of the response of Pseudomonas aeruginosa to hydrogen peroxide. J Bacteriol 186:248–252

Quadroni M, James P, Dainese-Hatt P, Kertesz MA (1999) Proteome mapping, mass spectrometric sequencing and reverse transcription-PCR for characterization of the sulfate starvation-induced response in *Pseudomonas aeruginosa* PAO1. Eur J Biochem 266:986–996

Roehl RA, Phibbs PV Jr (1982) Characterization and genetic mapping of fructose phosphotransferase mutations in Pseudomonas aeruginosa. J Bacteriol 149:897–905

Roehl RA, Feary TW, Phibbs PV Jr (1983) Clustering of mutations affecting central pathway enzymes of carbohydrate catabolism in Pseudomonas aeruginosa. J Bacteriol 156:1123–1129

Rutala WA (1996) APIC guideline for selection and use of disinfectants. 1994, 1995, and 1996 APIC Guidelines Committee. Association for Professionals in Infection Control and Epidemiology, Inc. Am J Infect Control 24:313–342

Salunkhe P, Topfer T, Buer J, Tummler B (2005) Genome-wide transcriptional profiling of the steady-state response of Pseudomonas aeruginosa to hydrogen peroxide. J Bacteriol 187:2565–2572

Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H (2003) Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. J Med Microbiol 52:403–408

Schraufstatter IU, Browne K, Harris A, Hyslop PA, Jackson JH, Quehenberger O, Cochrane CG (1990) Mechanisms of hypochlorite injury of target cells. J Clin Invest 85:554–562

Shimamoto G, Berk RS (1979) Catabolism of taurine in *Pseudomonas aeruginosa*. Biochim Biophys Acta 569:287–292

Shimamoto G, Berk RS (1980a) Taurine catabolism. II. Biochemical and genetic evidence for sulfoacetaldehyde sulfo-lyase involvement. Biochim Biophys Acta 632:121–130

Shimamoto G, Berk RS (1980b) Taurine catabolism. III. Evidence for the participation of the glyoxylate cycle. Biochim Biophys Acta 632:399–407

Spoering AL, Lewis K (2001) Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol 183:6746–6751

Tummler B, Kiewitz C (1999) Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. Mol Med Today 5:351–358

van der Ploeg JR, Weiss MA, Saller E, Nashimoto H, Saito N, Kertesz MA, Leisinger T (1996) Identification of sulfate starvation-regulated genes in *Escherichia coli*: a gene cluster involved in the utilization of taurine as a sulfur source. J Bacteriol 178:5438–5446

Vincent JL, Chierego M, Struelens M, Byl B (2004) Infection control in the intensive care unit. Expert Rev Anti Infect Ther 2:795–805

Weiss SJ, Klein R, Slivka A, Wei M (1982) Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. J Clin Invest 70:598–607

Winsor GL, Lo R, Sui SJ, Ung KS, Huang S, Cheng D, Ching WK, Hancock RE, Brinkman FS (2005) Pseudomonas aeruginosa Genome Database and PseudoCAP: facilitating community-based, continually updated, genome annotation. Nucleic Acids Res 33:D338–D343

Wylie JL, Worobec EA (1995) The OprB porin plays a central role in carbohydrate uptake in Pseudomonas aeruginosa. J Bacteriol 177:3021–3026

Zheng M, Wang X, Templeton LJ, Smulski DR, LaRossa RA, Storz G (2001) DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide. J Bacteriol 183:4562–4570