Global Transcriptomic Response of *Pseudomonas aeruginosa* to Chlorhexidine Diacetate

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Pseudomonas aeruginosa is implicated in nosocomial infections and chronic respiratory infections in cystic fibrosis patients. Chlorhexidine diacetate (CHX) is a biguanide disinfectant used for bacterial control in the hospital and agricultural and domestic environments. A better understanding of the mechanism of action of CHX and the resulting response elicited by *P. aeruginosa* to CHX will facilitate its effective utilization for *P. aeruginosa* control in these environments. This study presents, for the first time, the transcriptomic response of *P. aeruginosa* to 0.008 mM CHX after 10 and 60 min. Our results reveal that, after both treatment times, membrane transport, oxidative phosphorylation, and electron transport genes were downregulated. After 10 min, DNA repair was downregulated and the *opr*H gene that blocks the self-promoted uptake of antimicrobials was upregulated. After 60 min, outer membrane protein, flagellum, pilus, oxidative phosphorylation, and electron transport genes were downregulated. The *mex*C and *mex*D genes of the MexCD-OprJ multidrug efflux pump were significantly upregulated after both treatment times. The results of this study improve our understanding of the mode of action of CHX on *P. aeruginosa* and provide insights into the mechanism of action of other biguanide disinfectants which can be used for the development of more efficient disinfectants.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is of public health significance, as it is the most common cause of hospital-acquired (nosocomial) infections (*1*−*3*). Nosocomial infections are estimated to involve approximately 2 million patients each year, leading to 90 000 deaths and a huge economic impact of 4.5 billion dollars (*4*). *P. aeruginosa* has also been reported to contaminate disinfectants in the hospital environment, thereby compromising their purpose of reducing or eliminating bacterial contamination (*5*). In cystic fibrosis patients, *P. aeruginosa* causes chronic respiratory infections (*6*), and in a recent study, the home environment could not be ruled out as the source of new *P. aeruginosa* infections in cystic fibrosis patients (*7*). In poultry production, high early mortality rates of broiler chicks due to omphalitis have been associated with hatcheries contaminated by *P. aeruginosa* (*8*). With the increasing prevalence of antimicrobial and disinfectant-resistant pathogens, it is necessary that effective disinfecting strategies be employed, particularly in the hospital environment to counteract the growing problem of nosocomial infections.

Chlorhexidine is a cationic biguanide disinfectant that is available as the acetate, gluconate, and hydrochloride (*9*). Chlorhexidine diacetate (CHX) has been approved by the U.S. Environmental Protection Agency (EPA) for use as a disinfectant of agricultural and veterinary settings, egg handling and packing equipment, and meat and poultry processing plants. Chlorhexidine is also extensively used in hand washes, dressings and creams, instrument cleaning solutions, mouthwashes, and hospital disinfectant formulations (*10, 11*). Studies have shown that chlorhexidine may have applications in the formulation of antimicrobial mixtures used to control odor emissions from livestock waste, in the development of varnishes used for protecting bovine enamel and dentine, and in the development of antibacterial-releasing intrauterine devices to combat the development of pelvic infections (*12*−*14*). Although chlorhexidine has been found to be effective against a wide range of bacteria and some fungi and viruses, several hospital acquired gram-negative bacteria including *Pseudomonas*, *Klebsiella*, and *Serratia* are resistant to chlorhexidine (*9, 15*−*17*).

Previous studies have suggested that chlorhexidine treatment of *Pseudomonas* mainly affects the cell membrane, resulting in lysis and loss of cytoplasmic material (*18, 19*). Other studies have reported that the *P. aeruginosa* MexCD-OprJ efflux system of the resistance-nodulation-division (RND family) is inducible by some biocides including chlorhexidine (*2*). Despite these advances, the mechanisms responsible for chlorhexidine resistance in *P. aeruginosa* from a global genomic perspective have not been elucidated.

With such widespread use of chlorhexidine to combat bacterial contamination in different environments potentially harboring *P. aeruginosa*, it is important to determine the genetic basis of the cellular functions that it affects. This will enhance the understanding of the mode(s) of action of CHX, such that it can be more effectively utilized. Further, an improvement in the understanding of cellular protective processes utilized by *P. aeruginosa* to combat the inhibitory effects of CHX will enhance the development of more effective disinfectant treatments.

In this study, we investigated the global transcriptomic response of *P. aeruginosa* to treatment with 0.008 mM chlorhexidine diacetate for 10 and 60 min using Affymetrix *P. aeruginosa* GeneChip arrays. Real-time PCR was used to validate the microarray results. To our knowledge, this is the first study that documents the global transcriptomic response of *P. aeruginosa* to CHX. Given the widespread use of CHX as an antibacterial agent, the information from this study provides a useful resource for the understanding of its mechanism of action and the development of more effective CHX and biguanide based disinfectants.

Materials and Methods

Bacterial Growth and Treatment with CHX. Bacterial growth was carried out using methods previously described by our laboratory (*20, 21*). *P. aeruginosa* was grown in 100 mL of LB

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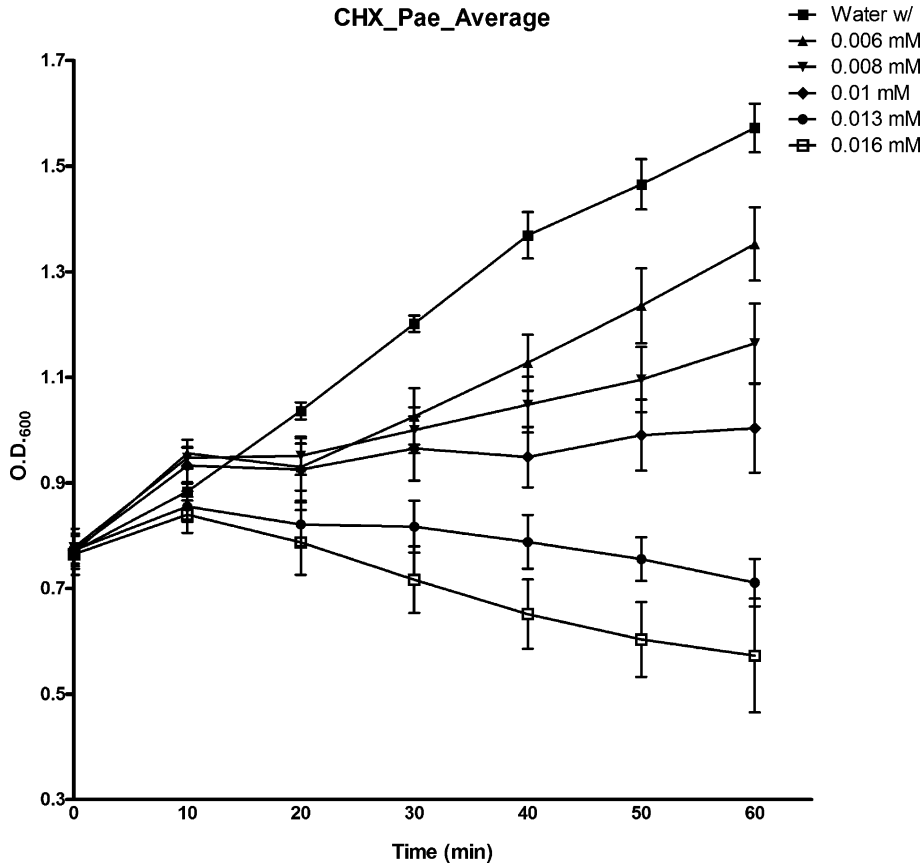

FIGURE 1. Growth inhibition of *P. aeruginosa* treated with chlorhexidine diacetate (CHX). Cell density was monitored as the OD_{600} in 10 min intervals. The CHX concentrations were as follows: 0 mM control with water (■), 0.006 mM (▲), 0.008 mM (▼), 0.01 mM (♦), 0.013 mM (●), and 0.016 mM (□). Each data point was derived as the average of three separate experiments, and the error bars represent the standard deviations obtained.

broth and incubated for 18−24 h at 37 °C with shaking at 250 rpm. A 1:100 dilution of the culture was performed using prewarmed LB broth. The diluted culture was incubated at 37 °C with shaking at 250 rpm until it reached an optical density (OD_{600}) of 0.8 (early logarithmic phase). A further 1:10 dilution was performed using LB broth, and the diluted culture was incubated at 37 °C with shaking at 250 rpm. When the OD_{600} of the 1:10 dilution reached 0.8, the culture was incubated at 37 °C with shaking at 250 rpm with different concentrations of CHX (Sigma-Aldrich, Inc., St Louis, MO), and the OD_{600} of the growth culture was determined at intervals of 10 min for a total time of 60 min.

RNA Isolation, cDNA Synthesis, Labeling, Hybridization, Staining, and Scanning. RNA isolation was carried out as previously described (*21*). cDNA synthesis, labeling, hybridization, staining, and scanning were carried out following Affymetrix protocols for *Pseudomonas aeruginosa* GeneChip arrays (Affymetrix, Inc., Santa Clara, CA).

Data Analysis. Data analysis was performed using the Affymetrix GeneChip Operating Software (GCOS), version 1.0 and GeneSpring Version 7.3 (Agilent Technologies). The following parameters were employed for expression analysis using GCOS: $\alpha_1 = 0.04$, $\alpha_2 = 0.06$, and $\tau = 0.015$, and target signal was scaled to 150. Genes that were assigned "absent calls" from 50% or more of the replicates in GeneSpring were not included in the analysis. Gene expression changes with statistical significance were identified by 1-way ANOVA (p cutoff value = 0.05). Fold changes were calculated as the ratios between the signal averages of three untreated (control) and three CHX-treated (experimental) cultures. Genes with a 2-fold or more induction or repression were used in this analysis.

Validation of Microarray Data Using Real-Time PCR. Quantitative real-time PCR on 20 genes was carried out in order to validate the transcript levels obtained by the microarray analysis. These genes were selected because they displayed a wide range of mRNA level changes (−3-fold to +14-fold). Table 2 indicates that our microarray results were in agreement with quantitative real-time PCR results. The real-time PCR results are the mean of three biological replicates with three technical replicates for each gene. The microarray results are the mean of three replicates of each gene.

Results and Discussion

Growth Inhibition of *P. aeruginosa* by CHX. In order to determine a suitable sublethal concentration of CHX that will produce strong growth inhibition, *P. aeruginosa* was treated with five concentrations of CHX dissolved in distilled water (0.006, 0.008, 0.01, 0.013, and 0.016 mM), and growth inhibition was monitored at intervals of 10 min for 60 min. In Figure 1, the highest concentration of CHX used (0.016 mM) produced drastic growth inhibition. Therefore, a lower sublethal concentration of 0.008 mM was selected as the test concentration since this concentration caused a nondrastic growth inhibition as seen in Figure 1.

Changes in the Transcriptional Profiles of *P. aeruginosa* in Response to CHX. Three microarray experiments were performed in the absence (control) and in the presence (experimental) of 0.008 mM CHX. To investigate early and late changes in transcription in response to CHX, RNA was isolated after 10 and 60 min exposure to 0.008 mM CHX. In order to determine which genes showed significant changes in the transcript level in response to CHX, the following

criteria were applied (i) the *p*-value for a Mann−Whitney test should be less than 0.05, (ii) an absolute fold change in transcript level should be equal to or greater than 2, and (iii) a gene should have a present or marginal call (Affymetrix, Inc.) from 50% or more replicates on both experimental and control replicate sets. After a one-way ANOVA was performed, 1197 out of the 5900 genes that make up the *P. aeruginosa* genome were statistically significant. Further analysis indicated that a total of 250 genes showed statistically marked upregulation (≥2-fold) or downregulation (≤2-fold) after a 10 min and after a 60 min exposure to CHX. The expression levels of the 5900 genes in the *P. aeruginosa* genome obtained from control experiments and after treatment with CHX (10 and 60 min) have been deposited in NCBI's gene Expression Omnibus (*22*) and can be accessed through the GEO series accession number GSE 14253.

Functional Classification of Upregulated and Downregulated Genes. In order to relate the up- and downregulated genes to their functions, the 250 statistically significant genes were placed into different functional classes. Functional classes were obtained from the *P. aeruginosa* Community Annotation Project (*23*). Figure S1 (in the Supporting Information) illustrates the grouping of up- and downregulated genes at 10 and 60 min into different functional classes and the total number of genes in each class for the two treatment times.

Figure S1 (in the Supporting Information) illustrates that, in general at 60 min, there were more downregulated genes in the functional classes, when compared to 10 min. In particular, genes belonging to the functional classes of "adaptation and protection", "energy metabolism", "fatty acid and phospholipid metabolism", and "membrane proteins" contained significantly more downregulated genes at 60 min compared to 10 min.

Among the upregulated genes, the functional classes of "antibiotic resistance and susceptibility", "chaperones and heat shock proteins", and "transcriptional regulation" contained more upregulated genes at 10 min compared to 60 min.

Grouping of Functionally Classified Up- and Downregulated Genes. To further analyze the 250 up- and downregulated genes, the genes were placed in six groups based on their transcription directions (Figure S2 in the Supporting Information). All of the genes discussed in this report can be found in Table S1 in the Supporting Information. However, for clarity and to facilitate the reading of this report, the genes discussed below in the six groups are indicated in Table 1.

Group I: Genes Upregulated after Both 10 and 60 min Exposure to CHX. The most upregulated gene in this group was PA4599 (*mex*C) with fold changes of approximately 14 after 10 min and 4 after 60 min. In addition, the *mex*D gene (PA4598) was upregulated approximately 6-fold after 10 min and 2-fold after 60 min of exposure to CHX. The *mex*CD-*opr*J operon encodes a multidrug efflux pump of the resistance nodulation-division (RND) family. This pump functions as an intrinsic determinant of antimicrobial resistance to several clinical antimicrobials (*24*−*26*), disinfectants, and other chemicals in *P. aeruginosa* (*2, 26*−*28*). Previous studies have shown that chlorhexidine disrupts the bacterial cell membrane (*18, 29*) and induces the expression of *mex*CD-*opr*J operon (*2, 30*). The upregulation of the MexCD-OprJ pump in this study, therefore, corroborates the results from previous studies that indicate that it plays a role in resistance to chlorhexidine in *P. aeruginosa*.

Group II: Genes Upregulated Only upon 10 min Exposure to CHX. Group II contained genes which were upregulated after 10 min but resumed normal transcription rates after a 60 min exposure to CHX. It has been established that, in *P. aeruginosa*, resistance to polycationic antimicrobials is attenuated due of the self-promoted uptake of these antimicrobials across the outer membrane (*31, 32*). The self-promoted uptake pathway involves the displacement of divalent cations that cross-link adjacent lipopolysaccharide molecules in the outer membrane by antimicrobial polycations (*33*). This causes distortions in the outer membrane, making the cell more permeable to the uptake of the antimicrobials. Previous studies have shown that that the overexpression of outer membrane protein H1 precursor (OprH) blocks the self-promoted uptake pathway (*34, 35*). In this study, the *opr*H gene was upregulated approximately 3-fold after 10 min of CHX exposure (Table 1). Considering that CHX is also a polycationic antimicrobial, this result suggests that the uptake of CHX through the self-promoted uptake pathway was repressed after 10 min, thereby functioning as an early protective response by blocking the transportation of CHX across the outer membrane.

A second upregulated gene in this group was *opr*J (Table 1), the multidrug efflux outer membrane protein precursor. OprJ is one of the proteins of the three-component multidrug efflux pump: MexCD-OprJ whose other two components (*mex*C and *mex*D) were upregulated after both treatment times (see group I). The MexCD-OprJ pump has been identified in *P. aeruginosa* as a determinant of intrinsic resistance to several clinical antimicrobials (*25*). A recent study demonstrated that chlorhexidine gluconate induces the expression of MexCD-OprJ in *P. aeruginosa*, implying that MexCD-OprJ plays an important role in innate multidrug resistance in wild-type *P. aeruginosa* in hospitals where disinfectants are frequently used (*2*).

The *nar*G gene (PA3875) that was also found in this group encodes the catalytic α subunit of the membrane-associated respiratory nitrate reductase, NarGHJI. In *E. coli*, the *nar*GHJI operon is induced during anaerobic growth when nitrate is used as a final electron acceptor instead of oxygen (*36*). These results are in agreement with previous studies from our laboratory which have shown that *P. aeruginosa* may switch to anaerobic respiration following treatment with peracetic acid (*37*) and ortho-phenylphenol (*21*).

Group III: Genes Downregulated Only upon 10 min Exposure to CHX. Group III contained genes which were downregulated after 10 min but resumed normal transcription rates after a 60 min exposure to CHX. The *nuo*D (NADH dehydrogenase I, chain C, D) and *nuo*F (NADH dehydrogenase I, chain F) genes, which are part of the NADH-coenzyme Q oxidoreductase (respiratory complex I) were downregulated in this group. This suggests that the mechanism of action of CHX may include the repression of oxidative phosphorylation as a route for energy production in *P. aeruginosa*.

The process of unwinding complementary strands of DNA, which is required by many features of DNA metabolism, is catalyzed by DNA helicase enzymes. The RecQ helicase family is highly conserved in evolution from prokaryotes to humans (*38*) and is essential for the maintenance of genome stability in all organisms (*39*). It has been suggested that RecQ performs replication fork repairs in cells unable to restart replication due to damaged replication forks (*40*). A recent study suggested that the formation of DNA−DNA cross-links and severe DNA breaks in pUC 18 plasmid DNA may be linked to the mechanism by which chlorhexidine-containing endodontic solutions kill root canal and periodontal microorganisms (*41*). The downregulation of the ATP-dependent DNA helicase (*rec*Q) after 10 min in this study, therefore, suggests that the downregulation of DNA repair may be contributory to the early growth inhibition in CHX-treated *P. aeruginosa* cells.

The *aspA* gene (PA5429) that encodes the aspartate ammonia−lyase enzyme was downregulated approximately 3-fold after 10 min of exposure to CHX (Table 1). Similar

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TABLE 1. List of Significantly Up- or Downregulated *P. aeruginosa* Genes

		10 min[a]		60 min[a]				
Affymetrix ORF #	probe ID	fold change[b]	*p* value	fold change[b]	p value	description	symbol	functional class
Group I: Upregulation (10 min), Upregulation (60 min)								
PA4599_mexC_at	PA4599	14.41	0.000719	3.80	0.000719	resistance-nodulation-cell division (RND) multidrug efflux membrane fusion protein MexC precursor	*mex*C	antibiotic resistance and susceptibility
PA4598_mexD_at	PA4598	5.81	0.00116	2.11	0.00116	resistance-nodulation-cell division (RND) multidrug efflux transporter MexD	*mex*D	antibiotic resistance and susceptibility
Group II: Upregulation (10 min), No Change (60 min)								
PA4597_oprJ_at	PA4597	2.13	0.00404			multidrug efflux outer membrane protein OprJ precursor	*opr*J	antibiotic resistance and susceptibility
PA1178_oprH_at	PA1178	3.29	0.000866			PhoP/Q and low Mg^{2+} inducible outer membrane protein H1 precursor	*opr*H	membrane proteins
PA3875_narG_at	PA3875	2.33	0.037			respiratory nitrate reductase alpha chain	*nar*G	energy metabolism
Group III: Downregulation (10 min), No Change (60 min)								
PA2639_nuoD_at	PA2639	−2.42	0.0022			NADH dehydrogenase I chain C,D	*nuo*D	energy metabolism
PA2641_nuoF_at	PA2641	−2.04	0.00155			NADH dehydrogenase I chain F	*nuo*F	energy metabolism
PA3344_recQ_at	PA3344	−2.26	0.022			ATP-dependent DNA helicase RecQ	*rec*Q	DNA replication, recombination, modification, and repair
PA5429_aspA_at	PA5429	−2.86	0.00518			aspartate ammonia-lyase	*asp*A	amino acid biosynthesis and metabolism
Group IV: No Change (10 min), Upregulation (60 min)								
PA2825_at	PA2825			2.70	0.0245	probable transcriptional regulator		two-component regulatory systems
Group V: No Change(10 min), Downregulation (60 min)								
PA2966_acpP_at	PA2966			−2.78	0.00711	acyl carrier protein	*acp*P	fatty acid and phospholipid metabolism
PA2968_fabD_at	PA2968			−2.55	0.0112	malonyl-CoA-[acyl-carrier-protein] transacylase	*fab*D	fatty acid and phospholipid metabolism
PA1609_fabB_at	PA1609			−2.48	0.00107	beta-ketoacyl-ACP synthase I	*fab*B	fatty acid and phospholipid metabolism
PA5174_at	PA5174			−2.26	0.039	probable beta-ketoacyl synthase		fatty acid and phospholipid metabolism
PA2965_fabF1_at	PA2965			−2.02	0.00406	beta-ketoacyl-acyl carrier protein synthase II	*fab*F1	fatty acid and phospholipid metabolism
PA0552_pgk_at	PA0552			−2.01	0.00894	phosphoglycerate kinase	*pgk*	carbon compound catabolism
PA1580_gltA_at	PA1580			−2.63	0.0194	citrate synthase	*glt*A	energy metabolism
PA4333_at	PA4333			−2.04	0.00341	probable fumarase		energy metabolism
PA4254_rpsQ_at	PA4254			−5.85	0.00339	30S ribosomal protein S17	*rpstt*Q	translation, post-translational modification, degradation
PA4255_rpmC_at	PA4255			−5.82	0.00496	50S ribosomal protein L29	*rpm*C	translation, post-translational modification, degradation
PA1556_at	PA1556			−5.93	0.00797	probable cytochrome c oxidase subunit		energy metabolism
PA1557_at	PA1557			−3.89	0.0279	probable cytochrome oxidase subunit (cbb3-type)		energy metabolism

TABLE 1. Continued

Affymetrix ORF #	probe ID	10 min[a] fold change[b]	10 min[a] *p* value	60 min[a] fold change[b]	60 min[a] p value	description	symbol	functional class
Group V: No Change(10 min), Downregulation (60 min)								
PA4430_at	PA4430			−3.89	0.00522	probable Cytochrome *b*		energy metabolism
PA5559_atpE_at	PA5559			−3.62	0.0037	atp synthase C chain	*atp*E	energy metabolism
PA5555_atpG_at	PA5555			−3.60	0.000668	ATP synthase gamma chain	*atp*G	energy metabolism
PA1583_sdhA_at	PA1583			−3.09	0.00146	succinate dehydrogenase (A subunit)	*sdh*A	energy metabolism
PA1581_sdhC_at	PA1581			−2.96	0.00502	succinate dehydrogenase (C subunit)	*sdh*C	energy metabolism
PA5554_atpD_at	PA5554			−2.95	0.000245	ATP synthase beta chain	*atp*D	energy metabolism
PA1584_sdhB_at	PA1584			−2.81	0.00168	succinate dehydrogenase (B subunit)	*sdh*B	energy metabolism
PA5556_atpA_at	PA5556			−2.71	0.0017	ATP synthase alpha chain	*atp*A	energy metabolism
PA1586_sucB_at	PA1586			−2.65	0.00194	dihydrolipoamide succiny ltransferase (E2 subunit)	*suc*B	energy metabolism
PA1580_gltA_at	PA1580			−2.63	0.0194	citrate synthase	*glt*A	energy metabolism
PA1582_sdhD_at	PA1582			−2.59	0.0022	succinate dehydrogenase (D subunit)	*sdh*D	energy metabolism
PA2644_nuoI_at	PA2644			−2.42	0.00182	NADH dehydrogenase I chain I	*nuo*I	energy metabolism
PA1555_at	PA1555			−2.36	0.0451	probable cytochrome c		energy metabolism
PA2952_etfB_at	PA2952			−2.32	0.00352	electron transfer flavoprotein beta-subunit	*etf*B	energy metabolism
PA1553_at	PA1553			−2.23	0.00155	probable cytochrome c oxidase subunit		energy metabolism
PA4333_at	PA4333			−2.04	0.00341	probable fumarase		energy metabolism
PA4429_at	PA4429			−2.00	0.00855	probable cytochrome c1 precursor		energy metabolism
PA2640_nuoE_at	PA2640			−2.00	0.00547	NADH dehydrogenase I chain E	*nuo*E	energy metabolism
PA0291_oprE_at	PA0291			−2.98	0.0008	anaerobically induced outer membrane porin OprE precursor	*opr*E	membrane proteins
PA0973_oprL_at	PA0973			−2.66	0.00803	peptidoglycan associated lipoprotein OprL precursor	*opr*L	membrane proteins
PA1777_oprF_at	PA1777			−2.61	0.0257	major porin and structural outer membrane porin OprF precursor	*opr*F	membrane proteins
PA3821_secD_at	PA3821			−2.46	0.000377	secretion protein SecD	*sec*D	membrane proteins
PA4525_pilA_at	PA4525			−6.11	0.000737	type 4 fimbrial precursor PilA	*pil*A	motility and attachment
PA5040_pilQ_at	PA5040			−2.45	0.0123	type 4 fimbrial biogenesis outer membrane protein PilQ precursor	*pil*Q	motility and attachment
PA1092_fliC_at	PA1092			−2.30	0.0134	flagellin type B	*fli*C	motility and attachment
PA1094_fliD_at	PA1094			−2.08	0.00198	flagellar capping protein FliD	*fli*D	motility and attachment
PA0409_pilH_at	PA0409			−2.23	0.0111	twitching motility protein PilH	*pil*H	chemotaxis
Group VI: Downregulation (10 min), Downregulation (60 min)								
PA4563_rpsT_at	PA4563	−7.56	0.000415	−2.92	0.000415	30S ribosomal protein S20	*rps*T	translation, post-translational modification, degradation
PA5570_rpmH_at	PA5570	−5.79	9.10×10^{-5}	−3.11	9.10×10^{-5}	50S ribosomal protein L34	*rpm*H	translation, post-translational modification, degradation
PA2851_efp_at	PA2851	−4.11	0.0104	−4.10	0.0104	translation elongation factor P	*efp*	translation, post-translational modification, degradation
PA5569_rnpA_at	PA5569	−3.34	0.00401	−3.31	0.00401	ribonuclease P protein component	*rnp*A	translation, post-translational modification, degradation
PA5128_secB_at	PA5128	−3.28	0.000763	−3.21	0.000763	secretion protein SecB	*sec*B	protein secretion/export apparatus
PA4276_secE_at	PA4276	−2.71	0.0015	−2.55	0.0015	secretion protein SecE	*sec*E	protein secretion/export apparatus
PA4243_secY_at	PA4243	−2.03	0.0063	−3.27	0.0063	secretion protein SecY	*sec*Y	membrane proteins

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TABLE 1. Continued

Affymetrix ORF #	probe ID	10 min[a] fold change[b]	10 min[a] *p* value	60 min[a] fold change[b]	60 min[a] p value	description	symbol	functional class
Group VI: Downregulation (10 min), Downregulation (60 min)								
PA4238_rpoA_at	PA4238	−2.20	0.000405	−3.42	0.000405	DNA-directed RNA polymerase alpha chain	*rpo*A	transcription, RNA processing and degradation
PA4275_nusG_at	PA4275	−3.29	0.00437	−2.60	0.00437	transcription antitermination protein NusG	*nus*G	transcription, RNA processing and degradation
PA5239_rho_at	PA5239	−2.14	0.00852	−2.27	0.00852	transcription termination factor Rho	*rho*	transcription, RNA processing and degradation
PA5015_aceE_at	PA5015	−2.45	0.0229	−3.50	0.0229	pyruvate dehydrogenase	*ace*E	amino acid biosynthesis and metabolism
PA2623_icd_at	PA2623	−2.30	0.00239	−2.38	0.00239	isocitrate dehydrogenase	*icd*	amino acid biosynthesis and metabolism
PA1588_sucC_at	PA1588	−2.16	0.00117	−3.48	0.00117	succinyl-CoA synthetase beta chain	*suc*C	energy metabolism
PA1589_sucD_at	PA1589	−2.14	0.000628	−2.89	0.000628	succinyl-CoA synthetase alpha chain	*suc*D	energy metabolism
PA2638_nuoB_at	PA2638	−2.68	0.00358	−2.41	0.00358	NADH dehydrogenase I chain B	*nuo*B	energy metabolism
PA5557_atpH_at	PA5557	−2.12	0.00617	−4.93	0.00617	ATP synthase delta chain	*atp*H	energy metabolism
PA5560_atpB_at	PA5560	−2.10	0.000519	−2.78	0.000519	ATP synthase A chain	*atp*B	energy metabolism
PA5558_atpF_at	PA5558	−2.01	0.00397	−4.63	0.00397	ATP synthase B chain	*atp*F	energy metabolism
PA1554_at	PA1554	−2.17	0.0243	−2.06	0.0243	probable cytochrome oxidase subunit (cbb3-type)		energy metabolism
PA4847_accB_at	PA4847	−2.76	0.000544	−3.20	0.000544	biotin carboxyl carrier protein (BCCP)	*acc*B	fatty acid and phospholipid metabolism
PA4848_accC_at	PA4848	−2.14	0.000366	−2.33	0.000366	biotin carboxylase	*acc*C	fatty acid and phospholipid metabolism
PA1610_fabA_at	PA1610	−2.38	0.00135	−2.52	0.00135	beta-hydroxydecanoyl-ACP dehydrase	*fab*A	fatty acid and phospholipid metabolism

[a] The microarray results are the mean of three replicates of each gene. [b] The fold change is a positive number when the expression level in the experiment increased compared to the control and is a negative number when the expression level in the experiment decreased compared to the control.

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TABLE 2. Transcript Level Comparison of *P. aeruginosa* Genes between Real-Time PCR and Microarray Analyses

	mRNA level change with microarray[a]		mRNA level change with real-time PCR[b]			
	fold change		fold change			
gene	10 min	60 min	10 min	60 min	forward primer sequence (5′-3′)	reverse primer sequence (5′-3′)
PA4599	14.41	3.80	25.00(±0.31)	6.67(±0.31)	TTTGCGTGCAATAGGAAGGATCGG	ACCTCCACCGGCAACACCATTT
PA4598	5.81	2.11	2.70(±0.17)	11.10(±0.31)	CGAATTCTTCATCAAGCGGCCGAA	AATTTGGAAATGACCAGCAGGCCG
PA4597	2.13		2.68(±0.52)		AACAGCAGCAACGAAGCCCTGAA	ATGAAGGCGATCTCGTTGAGGAAG
PA4600	3.64		12.5(±0.67)		TGATTTCCCATGACGAGCGACTCA	AGGCCTGGATGATCTGGTTCAGTA
PA3344	−2.26		−7.11(±0.17)		AGCTCAATTGCAGCGAGA AGA ACG	TCC TGG ATT TGC CGA GGA GTT
PA2639	−2.42		−6.06(±0.32)		TCAGCGTGTTCTACCACCTGATGT	TGATGCCGTACATGTCCCACACTT
PA5556		−2.71		−6.96(±1.51)	TACCGAGCTGATGAAGCAGAAGCA	TTCTCGTTGATCTTCGCCAGCAGA
PA2968		−2.55		−4.08(±1.53)	TCTGCATCCCTCGCATTCGTCTT	TTCAGGCGCTCTTCAGGACCATT
PA1777		−2.61		−3.81(±0.21)	ACTTCACCGAGAACTTCTTCGCCA	TTTCGAACCACCGAAGTTGAAGCC
PA1094		−2.08		−3.09(±0.32)	AACGAAGACATTCTCAAGGCGAGC	CGTACCGCTGTTGAACTTGGCATT
PA4386		−3.49		−4.92(±0.58)	TCCTCTGCATGATCGCGTCGTTAT	TTGTCCAGTACACGACCGGTACCTA
PA4243	−2.03	−3.27	−3.73(±1.01)	−4.92(±0.35)	ATGGCTAAGCAAGGTGCTCTCTCT	ACGATGATCGCCAGGAACAGGAAA
PA4275	−3.29	−2.60	−4.5(±0.47)	−2.63(±0.35)	GCTAAGCGTTGGTACGTTGTGCAT	ACCAGCACATAGCCAGGGAAGAAT
PA5015	−2.45	−3.50	−5.40(±1.72)	−8.00(±2.80)	AATGGTCAAGGACCTTTCCGACGA	TTCTTCACGTTGTGCGCGATGTTC
PA5560	−2.10	−2.78	−2.78(±1.29)	−2.14(±1.10)	AGAAACCGCTTCGGGTTACATCCA	AATGCCCAGAAGCCCATTTCCTTG
PA3001[c]	1.00	1.00	1.00	1.00	GCACCATCACCATCGACGAAGAAA	TCTTGATGCCGTACTGGGTGTAGT

[a] The microarray results are the mean of three replicates of each gene. [b] The real-time PCR results are the mean of three biological replicates with three technical replicates for each gene. [c] Internal control: glyceraldehyde-3-phosphate dehydrogenase.

results have been reported in *Escherichia coli*, where *aspA* expression was downregulated 22-fold after exposure to polyhexamethylene biguanide (PHMB) (*42*). Production of the AspA enzyme is induced under anaerobic conditions in order to synthesize fumarate as an electron acceptor (*43*). In contrast, the expression of *aspA* has also been reported to be upregulated under aerobic conditions (*44*). Other studies have reported that the expression of *aspA* is upregulated in *E. coli* in response to metal ion, acetate, and propionate treatment (*45, 46*). However, since both CHX and PHMB, both biguanide compounds, repressed the transcription of *aspA*, it is apparent that the expression of *aspA* may be differentially regulated depending on several factors that may include oxygen availability and the nature of the environmental stress.

Group IV: Genes Upregulated upon 60 min Exposure Only. Group IV consisted of eight genes whose expression levels were unchanged after 10 min and upregulated after 60 min of exposure to CHX. One gene in this group, PA2825, encodes a probable transcriptional regulator belonging to the functional class of two-component regulatory systems (Table 1).

Group V: Genes Downregulated upon 60 min Exposure Only. Group V contained genes which showed normal transcription rates after 10 min but were downregulated after a 60 min exposure to CHX. In this group, the most significant functional class was "translation, post-translational modification, and degradation" which contained 26 genes (Table S1 in the Supporting Information). The two most downregulated genes in this class are indicated on Table 1, and a complete list can be found in Table S1 in the Supporting Information. In line with the downregulation of translation was the repression of several amino acid (arginine, glycine, methionine, serine, and glutamine) biosynthesis genes with fold changes ranging from approximately −2 to −5.6 (Table S1 in the Supporting Information).

Group V of Table 1 also contains several genes involved in primary metabolic processes including fatty acid biosynthesis (*acp*P, *fab*B, D, G, and F1), the Embden-Meyerhof pathway (PA0552, phosphoglycerate kinase), and the citric acid (TCA) cycle (PA1580, citrate synthase; PA4333, fumarase). This group also contained two NADH dehydrogenase genes in complex I of the oxidative phosphorylation pathway which were uniformly downregulated approximately 2-fold. Succinate dehydrogenase genes in complex II were uniformly downregulated approximately 3-fold, a probable cytochrome c1 reductase precursor and other associated genes in complex III were downregulated approximately 2- to 4-fold, and a probable cytochrome c oxidase subunit and other associated genes in complex IV were downregulated approximately between 2- and 4-fold. Also, *atp*A, D, E, and G which encode components of the ATP synthase enzyme were uniformly downregulated approximately 3-fold (Table 1). The downregulation of these genes further supports the results of group III which indicate that CHX represses energy production through aerobic cellular respiration. These results are similar to those of a related study carried out in our laboratory which indicated that several genes of respiratory complexes I–IV were downregulated in *P. aeruginosa* treated with sodium hypochlorite (*20*). These results point to the possibility that the effects of oxidative disinfectants and biguanide disinfectants on energy metabolism in *P. aeruginosa* may be similar. However, more investigation is warranted to corroborate this theory.

The outer membrane of *P. aeruginosa* has a low level of permeability which provides innate resistance to several antimicrobial compounds (*33*). Outer membrane permeability is essentially determined by channel producing proteins (*47*). The *opr*D, E, L, and F genes that were downregulated approximately 2- to 4-fold (Table 1) encode outer membrane proteins that play roles in maintaining the

integrity of the outer membrane and control outer membrane permeability (*48*−*50*). The downregulation of these genes after 60 min in this study indicates that CHX treatment affects outer membrane permeability. These results are in agreement with other studies which indicate that polycationic disinfectants such as CHX target the outer membrane of *Pseudomonas* (*18, 19, 51*) that may lead to changes in outer membrane permeability.

In line with the downregulation of outer membrane proteins was the repression of genes encoding elements of flagella and pili, which are both outer membrane components. The flagellin type B (*fli*C) and the flagella capping protein (*fli*D) were downregulated approximately 2-fold after 60 min (Table 1), and flagella-mediated motility is an important determinant of virulence in *P. aeruginosa* (*52*). The *pil*A and the *pil*Q genes, which are involved in type IV pilus biogenesis, and the *pil*H twitching motility gene were downregulated approximately 6- and 2.5-fold, respectively (Table 1). The *pil*A and the *pil*Q genes have been shown to be essential for type IV pilus formation in *Pseudomonas* (*53, 54*). Type IV pili are necessary for surface colonization during biofilm formation and mediate twitching motility in *P. aeruginosa* (*53, 55, 56*). It has also been shown that the deletion of *pil*A in *P. stutzeri* leads to a loss of twitching motility capability (*53*). Considering that flagella-mediated motility, twitching motility, and multicellular behavior such as biofilm formation mediate virulence and cell survival in adverse environmental conditions, it is likely that the downregulation of these genes may be linked to growth inhibition in *P. aeruginosa* exposed to CHX.

Group VI: Genes Downregulated after Both 10 and 60 min Exposure to CHX. The most distinctive functional class in this group was "translation, post-translational modification, and degradation" which contained 33 genes (Table S1 in the Supporting Information). The two most downregulated 30 and 50S ribosomal proteins are indicated on Table 1. A complete list of ribosomal genes in this group can be found in Table S1 in the Supporting Information. The downregulation of these genes after both 10 and 60 min suggests that protein synthesis decreases in *P. aeruginosa* after a 10 min exposure to CHX and continues after prolonged exposure (60 min). In addition to the repression of protein synthesis genes, three genes of the Sec protein transport system, PA5128 (secB), PA4276 (*sec*E), and PA4243 (*sec*Y), were also downregulated after both treatment times. The SecY and SecE proteins are essential for the viability of the Sec translocon (*57*). The downregulation of both protein synthesis and protein transport genes suggests that protein transport across the cell membrane may be repressed following CHX treatment. In contrast, a recent study, indicated that *sec*Y, *sec*E, and *sec*G were upregulated in *P. aeruginosa* after both 20 and 60 min exposures to ortho-phenylphenol (*21*).

The downregulation of translation was concomitant with the repression of transcription-related genes in this group including the DNA-directed RNA polymerase alpha chain, PA4238 (*rpo*A), and a transcription antitermination factor, PA4257 (*nus*G). NusG has been shown to stimulate transcription antitermination through the N protein of phage λ (*58*). Further evidence indicating that transcription antitermination was repressed after both 10 and 60 min exposures of *P. aeruginosa* to CHX was the downregulation of PA5239 (*rho*). In *E. coli*, Rho-dependent transcription antitermination is modulated by NusG, which is an essential transcription elongation factor (*59, 60*).

As in group V, this group also contained several genes relating to the Embden-Meyerhof pathway and the TCA cycle including: the pyruvate dehydrogenase enzyme (*ace*E), the isocitrate dehydrogenase enzyme, (*icd*), and the succinyl-CoA synthetase enzymes (*suc*C and *suc*D). Further evidence supporting the repression of aerobic cellular respiration was the downregulation of several genes involved in oxidative phosphorylation, namely, the NADH dehydrogenase I chain B (*nuo*B) of the NADH-coenzyme Q oxidoreductase (respiratory complex I), PA1554 which is a probable cytochrome oxidase subunit of cytochrome c oxidase (respiratory complex IV), and *atp*H (PA5557), *atp*B (PA5560), and *atp*F (PA5558) which are all components of the bacterial ATP synthase (respiratory complex V).

The fatty acid biosynthesis genes, *acc*B (PA4847), *acc*C (PA4848), and *fab*A (PA1610), were downregulated after both treatment times. Both *acc*B and *acc*C play major roles in the acetyl-coA carboxylase complex that catalyzes the synthesis of malonyl-coA, which is the rate-limiting step of fatty acid biosynthesis (*61*), and the *fab*A gene plays a role in fatty acid elongation (*62*).

To our knowledge, this is the first study investigating the effect of CHX on *P. aeruginosa* from a global genomic perspective. We suspect that the upregulation of the *opr*H gene after 10 min, with normal transcription rates after 60 min, contributes to early resistance to CHX by blocking its translocation through the self-promoted uptake pathway into the cell. The significant upregulation of the MexCD-OprJ efflux pump after both treatment times in this study validates its function in the innate resistance to CHX in *P. aeruginosa*. The downregulation of outer membrane protein synthesis after 60 min may reflect changes in outer membrane permeability caused by CHX treatment. The downregulation of flagella and pili synthesis after 60 min, which are important determinants of virulence and environmental adaptation processes such as flagella-mediated motility, twitching motility, and pili-mediated biofilm may be related to the mode of action of CHX in *P. aeruginosa*. Furthermore, the results from this study indicate that a decrease in energy production after both treatment times in *P. aeruginosa* exposed to CHX through the downregulation of genes involved in oxidative phosphorylation and electron transport may also contribute to the growth inhibition caused by CHX. In summary, the mechanism of action of CHX in *P. aeruginosa* may be a multifaceted process involving changes in outer membrane permeability, the attenuation of virulence and environmental adaptation processes, and energy deprivation through the repression of genes involved in aerobic cellular respiration.

This gene expression profile can now be employed to more profoundly understand the mechanisms by which CHX inhibits *P. aeruginosa* growth and how this organism develops resistance to biguanide disinfectants in general and to CHX in particular. The information from this study provides useful information that will benefit further research on the toxicogenomic impact of biguanide disinfectants on *P. aeruginosa* and may contribute to the development of more effective disinfectants.

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Supporting Information Available

(1) Functional classification of statistically significant upregulated and downregulated genes after 10 and 60 min exposure to 0.008 mM CHX. (2) Classification of differentially regulated 250 genes into six groups based on their transcription directions after 10 and 60 min exposure to 0.008 mM

CHX. (3) List of 250 functionally classified *P. aeruginosa* genes that were significantly up- and downregulated after 10 and 60 min of CHX exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

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