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Genome-Wide Transcriptional Profiling of the Steady-State Response of *Pseudomonas aeruginosa* to Hydrogen Peroxide

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The steady-state response of *Pseudomonas aeruginosa* to the oxidative-stress-generating agent hydrogen peroxide was analyzed by PAO1 transcriptome profiling. In total, 694, 411, and 237 genes were upregulated and 668, 576, and 468 genes were downregulated in *P. aeruginosa* strains TB, 892, and PAO1, respectively. The expression profiles of the two variants of the TB clone were significantly more related to each other than the expression profile of either strain was to that of PAO1. Exposure to H_2O_2 activated by more than 10-fold the expression of the *cyoABCD* operon, which is key for aerobic respiration, and of oxidative-stress response elements such as the catalase KatB, the alkyl hydroperoxide reductase AhpF, and the thioredoxin reductase 2 operon. Genes for iron and sulfur homeostasis were upregulated. Most enzymes necessary for the conversion of amino acids into the citric acid cycle were globally downregulated at the transcriptional level. Nitrate respiration and arginine fermentation were shut off in the clone TB strains and attenuated in the PAO strain. The transcriptional profiles indicate that the two clone TB strains are more proficient in coping with H_2O_2-mediated oxidative stress than the reference strain PAO. According to this data, we recommend study of the transcriptome of strain PAO1 in parallel with those of at least two strains of another clone in order to differentiate common responses from clone- and strain-specific responses and to minimize overinterpretations of microarray data.

Pseudomonas aeruginosa is an increasingly prevalent opportunistic human pathogen and is the most common gram-negative bacterium found in nosocomial and life-threatening infections of immunocompromised patients (48). Patients with cystic fibrosis (CF) are especially disposed to *P. aeruginosa* infections, and for these persons the bacterium is responsible for high rates of morbidity and mortality (13, 26, 35). The bacterium synthesizes and secretes a number of virulence determinants that are postulated to enhance survival within the host by providing nutrients, evading the host immune response, and allowing the spread of the organism to other tissues. It has a remarkable ability to adapt to and persist in a variety of environments, including human hosts (15).

P. aeruginosa generates metabolic energy primarily through aerobic respiration. This process, involving a four-electron reduction of molecular oxygen (O_2) to water, can be potentially dangerous to the cell. Specifically, aberrant electron flow from the electron transport chain or cellular redox enzymes to O_2 can lead to the production of reactive oxygen intermediates (ROIs). These include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·). Furthermore, bacteria can be exposed to exogenous ROIs, especially during infection of humans, where phagocytes (e.g., neutrophils) mount a dramatic oxygen-dependent antimicrobial response (17, 30). The unchecked production or accumulation of these species can lead to cell damage, mutations, or death. *P. aeruginosa* possesses an impressive antioxidant armament for defense against ROIs, including two superoxide dismutases (SODs) cofactored by either iron (Fe-SOD) or manganese (Mn-SOD) (18, 20) to disproportionate O_2^- to H_2O_2 and O_2, three catalases (KatA, KatB, and KatC) (4, 27), and four alkyl hydroperoxide reductases (AhpA, AhpB, AhpCF, and Ohr) (34).

Polymorphonuclear-leukocyte-mediated phagocytosis and killing are the most important host defense mechanisms against *P. aeruginosa* (9). We were interested to know whether *P. aeruginosa* can evade oxidative stress and, if so, how. With limited exceptions, information regarding the nature and regulation of antioxidant enzymes in *P. aeruginosa* has been derived from studies of laboratory strains. There have not been extensive studies of these enzymes in clinical isolates of this organism. Recent technological advances have made it possible to study global gene expression in both prokaryotic and eukaryotic organisms by using high-density oligonucleotide microarrays. Completion of the entire *P. aeruginosa* PAO1 genome sequence (44), as well as the availability of high-density oligonucleotide arrays (Affymetrix *P. aeruginosa* GeneChip arrays), has permitted global gene expression profiling of *P. aeruginosa*. This approach has already been successfully used in studying *P. aeruginosa* gene expression under a number of different growth conditions (8, 10, 11, 33, 36, 39, 40, 41, 50). To compare the array data with those from the candidate gene approach (4, 19, 32, 34) and to compare the steady-state response with the acute-stress response (36), the model compound hydrogen peroxide was selected as the oxidative-stress-generating agent because it is a natural effector of innate immunity (30). The transcriptional profile of the sequenced reference strain PAO1, a burn wound isolate from the 1950s, was compared with that of the two clonal variants TB and 892, isolated from the lungs of patients with cystic fibrosis, which differ substantially in their intracellular-survival ability in poly-

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morphonuclear leukocytes (46). After exposure to hydrogen peroxide, a differential expression of the 1362, 987, and 705 genes was observed in strains TB, 892, and PAO1. The transcriptome data revealed a complex regulation of the cellular response to oxidative stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In order to assess hydrogen peroxide-mediated oxidative stress, three *P. aeruginosa* strains were selected: TB (TBCF10839), isolated from the sputum of a CF patient, Sehnde, Germany, in 1984; 892 (TBCF121838), isolated from the sputum of a CF patient, Hannover, Germany, in 1984 (47); and PAO1, the genomic and genetic reference strain isolated from a burn wound of a patient in Melbourne, Australia (44). *P. aeruginosa* TB and 892 belong to the same clone (47). All bacterial cultures were grown in 15 ml of Luria broth at 37°C at 300 rpm in 125-ml Erlenmeyer flasks to a turbidity of 5.0 (stationary phase) (optical density at 600 nm). The cultures grown to stationary phase (3×10^{10} cells) were resuspended in fresh Luria broth and kept in a dialysis tube (14-kDa cutoff; tube diameter, 25 mm) with an effective length of 6 cm for the exchange of fluids. Then the dialysis tube was resuspended in a 1-liter Erlenmeyer flask containing 600 ml of Luria broth without or with 10 mM hydrogen peroxide. The flasks were incubated at 37°C on a rotary shaker at 200 rpm for 2 h. After the incubation period, bacteria were immediately recovered from the dialysis tube and subjected to RNA isolation.

RNA isolation. The cells from the dialysis bags were recovered and harvested by centrifugation at 3,800 × *g* for 2 min at 4°C. Total RNA from approximately 3×10^{10} cells was extracted with a modified hot phenol method (45). Bacteria were quickly resuspended in 0.5 ml of distilled water and lysed in 7.5 ml of preheated (65°C) phenol-lysis buffer mix (5 ml of phenol [pH 5.5], 2.5 ml of 2% sodium dodecyl sulfate, 30 mM Na-acetate, 3 mM EDTA [pH 5.5]) with vigorous shaking for 10 min. The cell lysate was centrifuged at 3,800 × *g* for 20 min, and the supernatant was extracted with 3 ml of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and then subsequently with 3 ml of chloroform-isoamyl alcohol (24:1, vol/vol). To pellet the nucleic acids, 0.1 volume of 3 M Na-acetate (pH 5.2) and 2.5 volumes of ethanol were added and the mixture was incubated at −20°C overnight and centrifuged for 30 min at 3,800 × *g*. The pellet was washed with 5 ml of 70% ethanol and resuspended in 175 μl of diethyl pyrocarbonate-treated water. DNA was digested by the addition of 40 U of DNase I (Roche, Mannheim, Germany) and 20 U of SUPERaseIn (Ambion, Cambridgeshire, United Kingdom) in DNase I buffer (50 mM Na-acetate, 10 mM $MgCl_2$, 2 mM $CaCl_2$, pH 6.5) for 30 min at 37°C in a total volume of 200 μl. Then the RNA was purified with the use of RNeasy columns (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and the yield of total cellular RNA was quantified by measuring the light absorption at 260 nm. RNA with a size below 200 bp (e.g., tRNAs and 5S rRNA) is below the cutoff of the column and therefore could not be recovered. All the steps were carried out at 4°C, or RNA was also incubated on ice intermittently during the whole RNA isolation procedure. RNA samples were separated electrophoretically in 1.2% agarose with 2% formaldehyde as the denaturing reagent, and 1× MOPS (20 mM 4-morpholinepropanesulfonic acid, 10 mM Na-acetate, 1 mM EDTA, pH 7.0) buffer was used as the running buffer. The agarose gels were stained with ethidium bromide to check the purity and integrity of RNA preparation, with the 16S and 23S ribosomal bands as references.

Gene chip experiments and analyses. cDNA synthesis, fragmentation, and labeling and *P. aeruginosa* PAO1 gene chip hybridization and washing were performed according to the instructions of the manufacturer (Affymetrix, Santa Clara, Calif.) and as described by Salunkhe et al. (39). For each gene chip hybridization experiment, the culturing of bacteria, exposure to hydrogen peroxide, and subsequent RNA isolation were performed in duplicate on the same day, and the RNA preparations were then pooled for cDNA synthesis and hybridization onto a single GeneChip. A total of two GeneChips per strain and growth condition were scanned at 570 nm with a 3-μm resolution by the Affymetrix scanner. Data analysis was performed using Affymetrix Microarray Suite software 5.0 with Affymetrix default parameters. The average microarray hybridization signal intensity was scaled to 150. Two GeneChips per strain for growth in the presence and absence of hydrogen peroxide, respectively, were compared by the four-comparison survival method (2, 5). The data were imported into a Microsoft Access database capable of searching for genes, which were found in all four pairings that were (i) at least twofold regulated and (ii) defined by the Affymetrix Microarray Suite Software as having significant changes in their signal intensities. The arithmetic average and the standard deviation were calculated. Finally, a Bonferroni correction for multiple testing was applied as a rigorous criterion for significantly changed signal intensities (39). Data were combined with the annotation (dated 15 December 2004) from the website of the *P. aeruginosa* PAO1 sequence and the community annotation project provided at http://www.pseudomonas.com. Information about all significantly differentially expressed PAO genes in the three investigated strains is available at the authors' personal website (http://www.mh-hannover.de/kliniken/kinderheilkunde/kfg/pub.htm).

RESULTS AND DISCUSSION

Global microarray data analysis of the response of *P. aeruginosa* to hydrogen peroxide. DNA microarrays were used to study the global expression profile of *P. aeruginosa* in response to H_2O_2-mediated oxidative stress. Bacterial cultures grown in Luria broth to stationary phase were placed into a dialysis bag that was then placed in a 100-fold-larger surrounding volume of broth containing a bactericidal concentration of 10 mM hydrogen peroxide. Hence, a large discontinuous concentration gradient was established at the barrier of the dialysis membrane, generating a constant flow of hydrogen peroxide into the bag. By this procedure we allowed for an initial diffusion-controlled asymptotic increase in the H_2O_2 concentration until the steady state of influx and decomposition of H_2O_2 was established. This approach enabled most of the 3×10^{10} bacteria to adapt to the H_2O_2 stress and to remain viable. Prior to and after the 2-h exposure to 10 mM H_2O_2, numbers of CFU were the same (within the experimental error) for all three strains analyzed in this study, although a statistically nonsignificant trend to lower cell numbers was noted. In previous studies, the bacteria were typically directly exposed to H_2O_2 (16, 34, 36, 38, 52). The absence of any lag phase is of course a stronger perturbation of cell homeostasis and, depending on the H_2O_2 concentration, will evoke an immediate response ranging from virtually no reaction to 100% killing within seconds that is difficult to control. The separation of the bacteria from the stressor by a diffusion barrier enabled us to select a lethal concentration of H_2O_2 and hence to induce the maximal stress response in the bacteria without killing them. Moreover, H_2O_2 is notoriously unstable in aqueous solutions at 37°C, and although we started with 10 mM, a concentration of 7 to 8 mM, which is still lethal, was measured after 2 h. Hence, our experimental setting combined an initial asymptotic increase in stressor concentration with stationary maximal stress conditions thereafter. In summary, at the time point of 2 h, we could identify the specific steady-state response of *P. aeruginosa* to hydrogen peroxide.

According to the GeneChip analyses of the microarrays, the Pearson correlation coefficients were 0.98, 0.95, and 0.98 for the hydrogen peroxide-treated replicates and 0.45, 0.44, and 0.61 between hydrogen peroxide-treated and untreated control samples of TB, 892, and PAO1, respectively. The very low interexperimental variance of the results from replicates is reflected by the fact that after application of the Bonferroni correction for multiple testing of 5,900 independent items, all values indicating a 1.9-fold change or more in mRNA levels between H_2O_2-treated and untreated preparations from the same strain were classified as a significant change. After exposure for 2 h to hydrogen peroxide, 24, 17, and 12% of the 5,900 open reading frames were significantly differentially regulated in *P. aeruginosa* TB, 892, and PAO1, respectively. In total, 694, 411, and 237 genes were significantly upregulated and 668, 576,

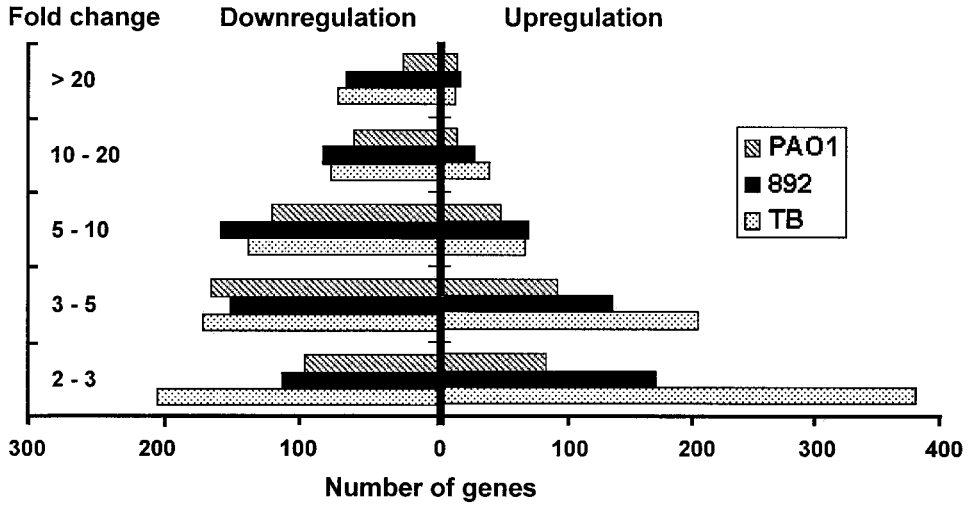

FIG. 1. Differential gene expression in response to hydrogen peroxide in the analyzed *P. aeruginosa* strains PAO1, 892, and TB. The number of genes showing increased or decreased expression and their levels of induction or repression are indicated.

and 468 genes were significantly downregulated in hydrogen peroxide-treated TB, 892, and PAO1 than in the untreated controls. Interestingly, the number of genes classified by the software as expressed differed by strain. Whereas more than 70% of genes were expressed by the TB and 892 strains in the presence or absence of hydrogen peroxide, the PAO1 strain expressed only 64% of genes in Luria broth medium. Exposure to hydrogen peroxide increased the proportion of expressed genes to 72%. Moreover, only 441 genes were consistently differentially expressed in the three strains. Seven hundred twenty-nine genes showed strain-specific regulation, and 501 genes were similarly regulated in two strains (Fig. 1). The expression profiles of the two TB clones were significantly more related to each other than they were to that of PAO1 ($P < 0.001$). The 892 strain shared 84 and 88% of up- and downregulated genes with its more virulent clonal variant TB, which exhibited a more strain-specific expression profile, with 50 and 76% of up- and downregulated genes being held in common with 892. It is noteworthy that strains TB and 892 exhibit identical informative single-nucleotide-polymorphism genotypes (31) and highly related SpeI and DraI macrorestriction fragment patterns (47). No nucleotide substitution was identified during comparative sequencing of 50 kb, and the sum of pulsed-field gel electrophoresis-separated SpeI fragment sizes yielded the same estimate of genome size (data not shown). The proteomes of the two strains are 96% identical under standard growth conditions in vitro (1). Nevertheless, the global mRNA expression profiles of the two strains were highly divergent when the strains were cultivated simultaneously under three different conditions (39; this work). In other words, genetically very closely related *P. aeruginosa* strains of matching genome sizes present strain-specific mRNA expression profiles that under carefully controlled identical conditions are highly reproducible but distinct from those of variants of the same clone. These individual signatures impede any generalizations about mRNA expression and gene regulation of the taxon. Hence, we chose two related isolates of a clone and the unrelated genetic reference strain PAO1 to get a clue about the relative impact of strain- and clone-specific responses versus general responses of *P. aeruginosa*.

The differentially regulated genes were classified by metabolic category (44). Genes encoding transporters and elements of amino acid metabolism were significantly overrepresented among the more strongly expressed genes in the presence of hydrogen peroxide, whereas the categories of chemotaxis, energy metabolism, and putative enzymes contained the relatively largest proportions of downregulated genes. The TB strain exhibited the largest number of upregulated genes in all but one category, whereas the patterns of downregulated genes were more similar among the three tested strains.

Major stress responses and changes in metabolic pathways to H_2O_2 treatment. Table 1 highlights the genes that changed consistently more than 10-fold in all three strains. Exposure to H_2O_2 activated the expression of the *cyoABCD* operon, which is key for aerobic respiration (6, 7), and of numerous determinants of the known elements of the oxidative-stress response (Table 2). The strongest induction was observed for the catalase KatB (PA4613), followed by alkyl hydroperoxide reductase AhpF (PA0140) and the thioredoxin reductase 2 operon (PA0848-PA0849). Constitutive high expression that increased by about a further 50% was observed for AhpC (PA0139), KatA (PA4236), and the superoxide dismutase SodB (PA4366). On the other hand, as already reported for the 10-min exposure to H_2O_2 (36), the OxyR-regulated genes *ahpB*, *katE*, *katN*, and *soxR* (34, 42, 43) were not induced by H_2O_2. Ohr, which directly detoxifies organic and inorganic hydroperoxides to less toxic metabolites (25), was 4- to 10-fold upregulated during continuous exposure to H_2O_2 (Table 2).

The oxidative-stress response is intimately linked with iron homeostasis. Fe^{2+} is essential for bacterial growth but also produces hydroxyl radicals from H_2O_2 that are regarded as the most potent nonselective oxidizing agent in biological systems (30). The bacteria managed to control iron homeostasis by increased expression of genes encoding the biosynthesis enzymes and receptor for the siderophore pyochelin (PA4221-PA4231, 3- to 26-fold upregulated), the hemin receptor

TABLE 1. Genes exhibiting a >10-fold change in mRNA levels in all three analyzed *P. aeruginosa* strains after exposure to hydrogen peroxide

PAO strain (gene name)	Avg fold change[a]			Protein name or description
	TB	892	PAO1	
Increased mRNA levels				
PA0140 (*ahpF*)	24	36	37	Alkyl hydroperoxide reductase subunit F
PA0848	16	25	32	Probable alkyl hydroperoxide reductase
PA0849 (*trxB2*)	16	23	33	Thioredoxin reductase 2
PA1317 (*cyoA*)	34	39	12	Cytochrome *o* ubiquinol oxidase subunit II; *cyoA*
PA1318 (*cyoB*)	18	28	20	Cytochrome *o* ubiquinol oxidase subunit I, *cyoB*
PA1320 (*cyoD*)	30	38	12	Cytochrome *o* ubiquinol oxidase subunit IV
PA3237	12	13	59	Hypothetical protein
PA4612	11	11	12	Conserved hypothetical protein
PA4613 (*katB*)	57	118	186	Catalase
Decreased mRNA levels				
PA0007	19	29	12	Hypothetical protein
PA0105 (*coxA*)	14	13	16	Cytochrome *c* oxidase; subunit II
PA0106 (*coxB*)	12	12	50	Cytochrome *c* oxidase; subunit I
PA0107	17	50	15	Conserved hypothetical protein
PA0109	12	15	11	Hypothetical protein
PA0122	35	19	10	Conserved hypothetical protein
PA0173	24	32	10	Probable methylesterase
PA0174	23	17	25	Conserved hypothetical protein
PA0176	25	23	16	Probable chemotaxis transducer
PA0177	23	26	12	Probable purine-binding chemotaxis protein
PA0178	17	16	13	Probable two-component sensor
PA0179	21	35	14	Probable two-component response regulator
PA0459	15	19	12	Probable ClpA or ClpB protease ATP binding subunit
PA0586	34	30	11	Conserved hypothetical protein
PA0587	28	22	11	Conserved hypothetical protein
PA0743	23	21	18	Probable 3-hydroxyisobutyrate dehydrogenase
PA1041	24	14	21	Probable outer membrane protein precursor
PA1172 (*napC*)	35	22	19	Cytochrome *c*-type protein NapC
PA1173 (*napB*)	40	68	15	Cytochrome *c*-type protein NapB precursor
PA1174 (*napA*)	36	23	17	Periplasmic nitrate reductase protein NapA
PA1175 (*napD*)	17	19	14	NapD protein of periplasmic nitrate reductase
PA1176 (*napF*)	27	29	17	Ferredoxin protein NapF
PA1177 (*napE*)	39	27	17	Periplasmic nitrate reductase protein NapE
PA1289	10	11	10	Hypothetical protein
PA1930	26	19	17	Probable chemotaxis transducer
PA2143	11	15	28	Hypothetical protein
PA2573	28	19	10	Probable chemotaxis transducer
PA2746	24	30	13	Hypothetical protein
PA2937	27	21	26	Hypothetical protein
PA3311	28	47	12	Conserved hypothetical protein
PA3415	30	23	38	Probable dihydrolipoamide acetyltransferase
PA3416	92	47	103	Probable pyruvate dehydrogenase E1 component; beta chain
PA3417	31	26	42	Probable pyruvate dehydrogenase E1 component; alpha subunit
PA3418 (*ldh*)	29	28	22	Leucine dehydrogenase
PA3520	39	23	23	Hypothetical protein
PA3723	17	13	14	Probable flavin mononucleotide oxidoreductase
PA3870 (*moaA1*)	24	27	17	Molybdopterin biosynthetic protein A1
PA3871	35	68	23	Probable peptidyl-prolyl *cis-trans* isomerase; PpiC type
PA3872 (*narI*)	37	50	18	Respiratory nitrate reductase gamma chain
PA3873 (*narJ*)	55	77	22	Respiratory nitrate reductase delta chain
PA3874 (*narH*)	79	305	25	Respiratory nitrate reductase beta chain
PA3875 (*narG*)	273	415	21	Respiratory nitrate reductase alpha chain
PA3876 (*narK2*)	62	71	12	Nitrite extrusion protein 2
PA3877 (*narK1*)	20	64	11	Nitrite extrusion protein 1
PA3986	11	12	11	Hypothetical protein
PA4296	11	14	13	Probable two-component response regulator
PA4306	59	28	64	Hypothetical protein
PA4573	14	13	12	Hypothetical protein
PA4607	19	10	10	Hypothetical protein
PA4648	28	14	11	Hypothetical protein
PA4651	31	14	15	Probable pilus assembly chaperone
PA4702	12	12	13	Hypothetical protein
PA4703	10	12	16	Hypothetical protein
PA5435	18	17	14	Probable transcarboxylase subunit
PA5436	10	12	14	Probable biotin carboxylase subunit of a transcarboxylase

[a] Values are means of results of four independent RNA preparations.

TABLE 2. Expression of mRNA transcripts known to be involved in the oxidative stress response

Gene	PAO strain	GeneChip normalized signal intensity[a]					
		Strain TB		Strain 892		Strain PAO1	
		−	+	−	+	−	+
ahpC	PA0139	2,320	3,894	1,989	3,948	2,282	8,751
ahpF	PA0140	63	1,650	57	1,473	86	2,662
ahpB	PA0847	13	4	17	5	112	19
	PA0848	66	1,212	51	1,502	54	1,405
trxB2	PA0849	51	1,227	46	1,510	73	2,393
gor	PA2025	392	330	270	305	174	360
katE	PA2147	4	4	3	3	18	5
katN	PA2185	11	12	11	5	12	7
soxR	PA2273	10	6	2	6	24	55
ohr	PA2850	56	201	45	273	46	464
bfrB	PA3531	2,224	1,710	2,998	1,495	2,667	459
bfrA	PA4235	1,964	710	1,791	724	1,520	1,144
katA	PA4236	1,770	2,374	1,510	2,363	3,712	5,742
sodB	PA4366	1,490	2,053	1,434	2,188	1,121	2,014
sodM	PA4468	30	75	24	45	52	112
fumC1	PA4470	44	156	27	115	44	197
ankB	PA4612	13	84	12	80	38	521
katB	PA4613	23	1,736	12	1,782	16	2,798
oxyR	PA5344	229	378	252	398	256	388

[a] Signal intensities were measured in the absence (−) and presence (+) of 10 mM hydrogen peroxide.

TABLE 3. Expression of mRNA transcripts involved in microaerophilic and anaerobic growth

Gene	PAO strain	GeneChip normalized signal intensity[a]					
		Strain TB		Strain 892		Strain PAO1	
		−	+	−	+	−	+
nirN	PA0509	1,537	28	1,627	46	1,578	637
	PA0510	1,545	5	1,369	14	1,202	410
nirJ	PA0511	1,813	32	1,680	52	2,459	830
	PA0512	1,656	12	1,373	25	962	371
	PA0513	1,503	8	1,403	27	1,023	361
nirL	PA0514	1,504	6	1,393	27	2,144	726
	PA0515	2,297	3	2,198	49	2,702	960
nirF	PA0516	2,319	18	2,070	48	2,014	676
nirC	PA0517	3,080	16	3,045	100	3,878	1,391
norC	PA0523	3,366	32	3,045	130	1,983	363
norB	PA0524	2,666	8	2,348	35	959	218
	PA0525	1,143	13	981	14	1,348	315
nosR	PA3391	586	6	554	27	639	170
nosZ	PA3392	2,332	22	2,555	47	3,145	1,520
nosD	PA3393	674	31	853	36	562	233
nosF	PA3394	338	10	386	10	412	168
moaA1	PA3870	278	11	482	8	558	31
	PA3871	524	10	697	10	1,589	59
narI	PA3872	994	29	1,754	23	3,306	204
narJ	PA3873	1,016	14	1,774	25	1,880	81
narH	PA3874	1,135	20	1,984	15	1,908	75
narG	PA3875	1,008	2	1,697	12	1,208	50
narK2	PA3876	686	11	1,202	25	4,925	42
narK1	PA3877	785	63	1,309	47	1,106	96
arcA	PA5171	4,057	200	3,885	185	9,525	4,190
arcB	PA5172	4,842	79	4,652	178	11,199	4,754
arcC	PA5173	3,465	37	3,651	81	5,571	2,216

[a] Signal intensities were measured in the absence (−) and presence (+) of 10 mM hydrogen peroxide.

(PA4664, 3-fold upregulated), and the HitAB iron binding and transport system (PA4687-PA4688, 2- to 8-fold upregulated), and they controlled the downregulation of bacterioferritins (2- to 9-fold downregulated). Moreover, individual strains upregulated heme biosynthesis, iron-sulfur proteins, and iron transport systems. Another important element affected by oxidative stress is sulfur. On one hand, free thiol groups are oxidized to disulfide bonds, and on the other, sulfur is an essential part of iron-sulfur proteins that are particularly prone to oxidative damage (23). In particular, the two strains of the TB clone were proficient in activating the sulfate transport operon cysAWT, the sulfate binding proteins Sbp and CysP, sulfite reductase CysI, and the ATP sulfurylase CysND to supply sulfur to the cell. Sulfur was kept as reduced thiol by high levels of thioredoxin reductase.

Besides the direct impact of H_2O_2 on the activation of direct defense mechanisms, the strong stationary exposure to this detrimental agent caused responses in primary metabolism and signal transduction. Numerous transcriptional regulators became differentially expressed; in particular, two-component systems associated with chemotaxis were downregulated, e.g., PA0174-PA0180 (7- to 25-fold) and PA2571-PA2573 (4- to 28-fold). Most enzymes necessary for the conversion of amino acids into the citric acid cycle were globally downregulated at the transcriptional level. Nitrate respiration and arginine fermentation were shut off in the clone TB strains and attenuated in the PAO strain. Table 3 lists these strikingly different levels of 2- to 500-fold downregulation of mRNA transcripts of the operons PA0509-PA0517, PA0523-PA0525, PA3391-PA3394, PA3870-PA3877, and PA5171-PA5173, which represent the major pathways of anaerobic energy metabolism in *P. aeruginosa* (53). Anaerobic respiration requires molybdenum as a cofactor for catalytic activity (53), and correspondingly, the elements for molybdenum transport and molybdopterin biosynthesis were significantly less expressed in the presence of H_2O_2.

Reactive oxygen intermediates cause damage to nucleic acids and proteins. Accordingly, numerous elements of DNA repair, such as RecA, RecR, LexA, MutS, and DnaX, were upregulated at the transcriptional level. Protein biosynthesis was maintained by stronger expression of mRNAs for ribosomal proteins and aminoacyl-tRNA-synthetases, and in parallel, the ribosome modulation factor that inactivates the ribosome (51) was downregulated. Several genes encoding prolyl-peptidyl isomerases were more strongly transcribed to reverse damage by misfolding. *clpA* protease genes were less transcribed to ensure a decreased rate of protein degradation. In summary, these data indicate that the bacteria tried to keep the mutation rate and the loss of protein to a minimum.

Exposure to H_2O_2 also affected secondary metabolism and major pathways of bacterial virulence. The three tested *P. aeruginosa* strains exhibited lower mRNA levels of genes encoding the biosynthesis of the PQS signal (37), effector proteins of type I and type II secretion (24), and extracellular polymers, such as exopolysaccharides (29) and polyhydroxyalkanoic acid (21).

Besides these responses common to all three investigated strains, multiple clone- and strain-specific responses were observed (Fig. 2). Each strain activated other members of the extracytoplasmic-function σ^{70} family of global transcription factors (from strain PAO1, PA0472; from TB, PA1300; and

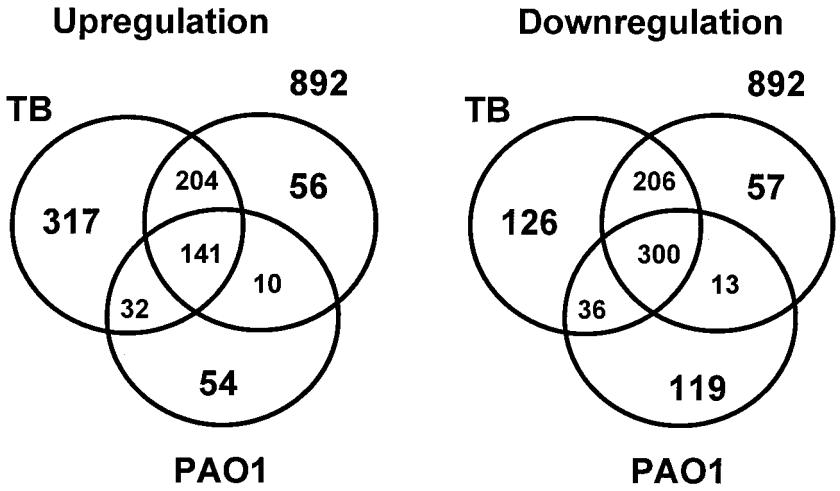

FIG. 2. PAO1 genes with significantly changed expression levels in hydrogen peroxide-treated *P. aeruginosa* TB, 892, and PAO1. Genes that were found to be up- or downregulated in two or three strains are indicated in the intersections.

from 892, PA2387 and PA3285) (28). Strain PAO1, but not the clone TB strains, induced the expression of the FtsYE cell division operon (PA0373-PA0374) and reduced the expression of the global transcriptional regulator GacA and numerous elements in the degradation and secondary metabolism of aromatic amino acids. The expression of a total of 13 ABC transporters, symporters, efflux systems, and permeases was downregulated in strain PAO1. With respect to the expression of transporters, an opposite response of the two clone TB strains was observed. The TB and 892 strains increased the mRNA levels for 23 different transport systems, including five ABC transporters, the MexEFOprN efflux pump, the Na^+/H^+ antiporter, and transporters or permeases for citrate, lactate, and amino acids.

Exposure to H_2O_2 had a major impact on energy metabolism at the transcriptional level. Levels of cytochrome *c* oxidase mRNA transcripts (PA0105-PA0108 and PA1555-PA1557) in one of the terminal energy-transducing respiratory complexes were moderately (PA1555-PA1557, 2- to 13-fold) or strongly (PA0105-PA0108, 7- to 50-fold) reduced in the presence of H_2O_2. Alternative routes of anaerobic energy metabolism to synthesize ATP were also downregulated. Strain PAO reduced the concentration of all mRNA species of nitrate respiration. Strains 892 and TB reacted even more strongly to H_2O_2 by complete elimination of nitrate respiration and acetate and arginine fermentation. As alternatives for ATP synthesis, two systems were upregulated: the *cyoABCDE* operon (PA1317-PA1321), encoding cytochrome *o* ubiquinol oxidase, the main terminal oxidase of the electron transport chain under highly aerobic conditions (14), and the *nqrFEDCBA* operon (PA2994-PA2999), encoding the Na^+-translocating NADH-quinone oxidoreductase (3, 12), which can probably function as complex I of the respiratory chain, by which it pumps sodium ions instead of protons (12).

Comparison with published transcriptome studies of the response of *P. aeruginosa* to oxidative stress. Exposure to hydrogen peroxide influenced the expression of hundreds of *P. aeruginosa* genes belonging to numerous metabolic categories. The damaging hydroxyl radicals are produced not only by decomposition of hydrogen peroxide but also by superoxide. The superoxide anion is an unavoidable by-product of aerobic metabolism (22). It leaches free iron from iron-sulfur clusters and promotes hydroxyl-radical formation and consequent DNA damage. We previously measured the genome-wide transcription profile against the superoxide-generating agent paraquat in the same *P. aeruginosa* strains employed in this study, PAO1, TB, and 892 (39). One operon of conserved hypotheticals (PA0939-PA0942) was consistently 20- to 200-fold upregulated in the three strains, but otherwise only 24 to 29 subtle strain-specific changes in the oxidative-stress response (*ahpF*), energy metabolism (*cox* operon), iron-responsive elements, and transport were noted. Of these strain-specific responses, all of the 17 paraquat-repressed genes and 13 of the 38 paraquat-induced genes were also down- and upregulated by the exposure to hydrogen peroxide. Hence, paraquat modulates a small subset of the genes that are differentially regulated by hydrogen peroxide. However, with the exception of a single operon, no changes in the mRNA expression pattern common to all three strains occurred when the bacteria were exposed to 0.5 mM paraquat for 2 h under the same experimental conditions as applied here. We would like to conclude that in our experimental setup, hydrogen peroxide with its massive production of hydroxyl radicals and oxygen more strongly affects metabolism and signaling in *P. aeruginosa* than the superoxide anion.

We report here on the steady-state response to hydrogen peroxide, whereas Palma and colleagues (36) have analyzed the global transcriptional profile of the early adaptation phase. *P. aeruginosa* PAO1 bacteria were directly exposed to a sublethal concentration of 1 mM hydrogen peroxide for 10 min. Only 60 of 2,525 genes detected as significantly differentially regulated in one or the other study were consistently up- or downregulated in both transcriptome analyses (Fig. 3). Two hundred eleven genes were discordantly regulated in the two studies. All genes but the *cyo* operon that were found to be consistently upregulated in our study by more than 10-fold in all three strains (Table 1) were also reported by Palma et al. (36) to be strongly activated. The key players of an inducible oxidative-stress response, such as alkylhydroperoxide reduc-

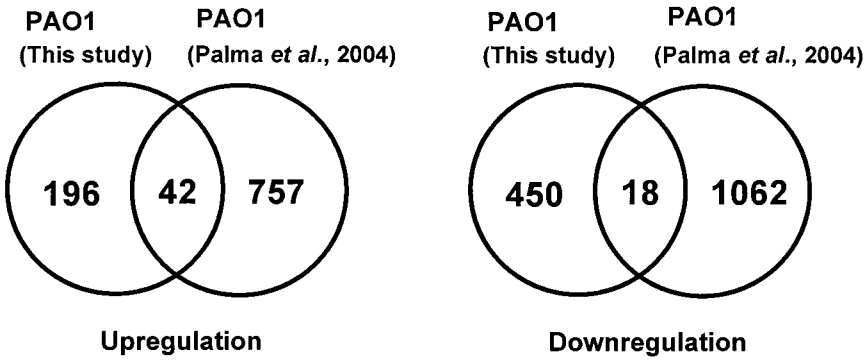

FIG. 3. Global comparison of the genes identified to be differentially regulated upon exposure to hydrogen peroxide by Palma et al. (36) and in this study.

tase, catalase, and thioredoxin reductase (Table 2), were highly expressed in the PAO1 strain under both experimental conditions. In contrast, the overlap of the subset of the 55 consistently >10-fold-downregulated genes in our study with the complete set of 1,080 downregulated PAO1 genes in Palma's study is nil. The low similarity of the transcriptome profiles suggests that *P. aeruginosa* copes differently with hydrogen peroxide-induced stress during the initial adaptation and the later stationary phase. However, as already outlined in an article in this journal (49) on the quorum-sensing transcriptome of PAO1 (41, 50), differential handling and growth conditions of PAO1 cells and differences in the genotypes and phenotypes of the PAO1 strain (44) may also account for divergent mRNA transcription data. Hence, we analyzed in parallel three strains in order to differentiate between common responses and clone- and strain-specific responses and thereby to identify the signature of the species-typical response to the stressor (Fig. 2). To date when independent investigators have studied the PAO1 transcriptome with the same or similar objectives, discordant profiles have been obtained, and we recommend for future transcriptome studies that PAO1 and at least two strains of another clone always be incorporated in order to estimate more reliably inherent and experimental variances.

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