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Transcriptome Analysis of the Response of *Pseudomonas aeruginosa* to Hydrogen Peroxide

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Pseudomonas aeruginosa **must often overcome a high concentration of oxidants to successfully infect the human host. We report here the results of a transcriptome profiling comparing cells treated with H_2O_2 and untreated controls. The data indicate that the early response of *P. aeruginosa* to H_2O_2 consists of an upregulation of protective mechanisms and a downregulation of primary metabolism.**

The 6.3-Mb genome of *Pseudomonas aeruginosa* is one of the largest bacterial genomes sequenced (28). In addition to having a free lifestyle, *P. aeruginosa* causes opportunistic infections in humans (7). Studies with animals suggest that an adaptive mechanism important for the ability of *P. aeruginosa* to infect humans is the oxidative stress response (7). This response is aimed at preventing, counteracting, and repairing oxidative damage produced by reactive oxygen intermediates (ROIs) such as H_2O_2, $O_2^{\bullet -}$, and $OH^{\bullet}$. During infection, *P. aeruginosa* is confronted with ROIs from the respiratory burst of human phagocytes (19). In particular, the inflammatory response in the infected lungs of cystic fibrosis patients results in high levels of ROIs that *P. aeruginosa* must survive to persist (2). Several *P. aeruginosa* proteins involved in oxidative stress defense have been identified (3, 6, 13, 18), and some of these proteins have been shown to be induced by treatment with oxidants (see below).

To gain insights into the early transcriptional response of *P. aeruginosa* to oxidative stress, we performed a comparative transcriptome analysis between H_2O_2-treated *P. aeruginosa* PAO1 cells and untreated controls by using GeneChip *P. aeruginosa* genome arrays (Affymetrix). H_2O_2 was selected as the oxidant because it is a natural effector of innate immunity (19). Two milliliters of an overnight culture was inoculated into a 1-liter Erlenmeyer flask with 100 ml of tryptic soy (TS) broth (11), and cultures were incubated at 37°C with shaking at 220 rpm. When the optical density at 600 nm reached 0.5 (0.5×10^8 to 1.0×10^8 CFU/ml), the culture was split in aliquots of 10 ml into 100-ml Erlenmeyer flasks. Five of these cultures were immediately treated by addition of H_2O_2 (1 mM), whereas the remaining five cultures were left untreated. After 10 min of incubation under the conditions mentioned above, cells from each culture were harvested by centrifugation and RNA was isolated from each cell pellet by using the RNeasy Minikit (Qiagen). cDNA was prepared from each RNA sample by using $pd(N)_6$ random primers and the SuperScript II kit (Invitrogen). Each cDNA sample was fragmented (average size, 50 bp) with DNase I in One Phor-All buffer (Invitrogen) at 37°C for 10 min, and fragmented cDNA was 3′ labeled by using the Enzo BioArray Terminal kit with Bioin-ddUTP (Affymetrix). Each labeled cDNA sample was used for hybridization to a single *P. aeruginosa* array. Array hybridization and array and data analysis were performed as recommended in the GeneChip *P. aeruginosa* genome array expression analysis protocol technical manual (Affymetrix). A 1 mM H_2O_2 concentration was selected for treatment, since it is commonly used to trigger sublethal oxidative stress in *P. aeruginosa* (12, 18). A 10-min treatment time was chosen based on several considerations. (i) A time span of 5 to 15 min is sufficient to detect mRNA level changes induced by H_2O_2 in *P. aeruginosa*, *Escherichia coli*, and other bacteria (1, 2, 22, 25). (ii) Our reverse transcription (RT)-PCR studies revealed that *sodM* mRNA levels increase after a 10-min exposure to 1 mM H_2O_2 but return to nearly the levels of the untreated control after 20 min (not shown). (iii) H_2O_2 is rapidly degraded in *P. aeruginosa* cultures due to endogenous catalases; e.g., H_2O_2 in TS broth drops at a rate of ~0.17 mM/min during a 15-min period after addition of only 20% *P. aeruginosa* spent medium (15). (iv) We wanted to investigate the early and acute transcriptional response to H_2O_2.

Transcriptome changes induced by H_2O_2. A global analysis indicated that the early response of *P. aeruginosa* to H_2O_2 resulted in a substantial modification of the transcriptome. Of 5,500 arrayed *P. aeruginosa* genes (open reading frames), 1,854 displayed statistically significant mRNA level changes of >2-fold (genes with statistically significant expression changes were identified by the Wilcoxon-Mann-Whitney test [*P* cutoff value, 0.05]) and 2,792 displayed no change. The latter group was defined as being composed of genes showing no significant mRNA level changes and genes with statistically significant changes of twofold or less. Finally, mRNAs of 854 genes were not detected above background in three or more of the five arrays of treated and untreated samples. Substantial changes of mRNA levels (≥5-fold) were observed for 520 genes, with 216 of them displaying increased transcript levels (Table 1). Interestingly, genes in the functional classes of "hypothetical, unclassified, unknown" and "transport of small molecules" were highly represented in this group (Fig. 1). (Gene names, descriptions, and functional classes are taken from http://www.pseudomonas.com/current_annotation.asp.) This response is

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TABLE 1. Effects of treatment with H_2O_2 on mRNA levels

Change in mRNA level	No. of genes (% of total) with indicated mRNA level change[a]			
	≥5-fold	≥5- to 10-fold	>10- to 50-fold	>50-fold
Increase	216 (3.9)	133 (2.4)	76 (1.4)	7 (0.1)
Decrease	304 (5.5)	247 (4.5)	57 (1.0)	0 (0.0)
Total	520 (9.4)	380 (6.9)	133 (2.4)	7 (0.1)

[a] Change was calculated as the ratio of the means of normalized gene signals of treated to those of untreated cultures.

comparable to that of *E. coli* exposed to identical treatment (1 mM H_2O_2, 10 min), which resulted in 140 of 4,169 arrayed genes displaying mRNA levels increased >4-fold (32).

Recently, Salunkhe and coworkers reported *P. aeruginosa* transcriptome profiling aimed at studying the steady-state response, rather than the early adaptation phase, to paraquat (0.5 mM, 2 h) (27). Unexpectedly, induction of various genes (e.g., *katB*, *soxR*, *ahpC*, *trxB2*, *ohr*, and *gor*) shown in our array study and by other investigators to be induced by oxidant treatment was not observed in this study for yet unclear reasons (see below). Only 21 to 29 gene transcripts showed significant change (≥2-fold) with treatment in each of the three strains investigated. This represented a total of 55 genes after considering the overlaps between strains. mRNA levels of 29 of these 55 genes were affected in our study by H_2O_2 treatment (PA0105 to 0107, PA0140, PA0672, PA0848, PA0941, PA0942, PA1300, PA2033, PA2230, PA2426, PA2663, PA3234, PA3235, PA3397, PA3417, PA3899, PA4205, PA4227, PA4296, PA4467 to PA4471, PA4502, PA4504, and PA4570).

Genes whose mRNA levels increased with H_2O_2 treatment. Treatment with H_2O_2 resulted in a dramatic increase in the mRNA levels of many genes that, based on their known or suspected functions, would prevent, counteract, or repair oxidant-derived damage. These genes appear to be part of two main regulons, an oxidant-responsive regulon, whose genes are likely to be directly induced by H_2O_2, and the SOS regulon, whose genes are likely to be induced by the expected oxidant-dependent DNA damage. Some of the genes displaying mRNA level increases are listed in Table 2. Additional tables that can be found at http://www.med.cornell.edu/gradschool/fac/quadri.html (site under construction) include the complete lists of genes with statistically significant mRNA level increases (Supplemental Table 1) and decreases (Supplemental Table 2), the list of genes not detected in the arrays (Supplemental Table 3), and the entire data sets for the arrays (Supplemental Table 4).

The mRNA levels of many genes relevant for oxidative stress adaptation increased dramatically with treatment. Examples of mRNAs with important level changes (≥8-fold) are those of

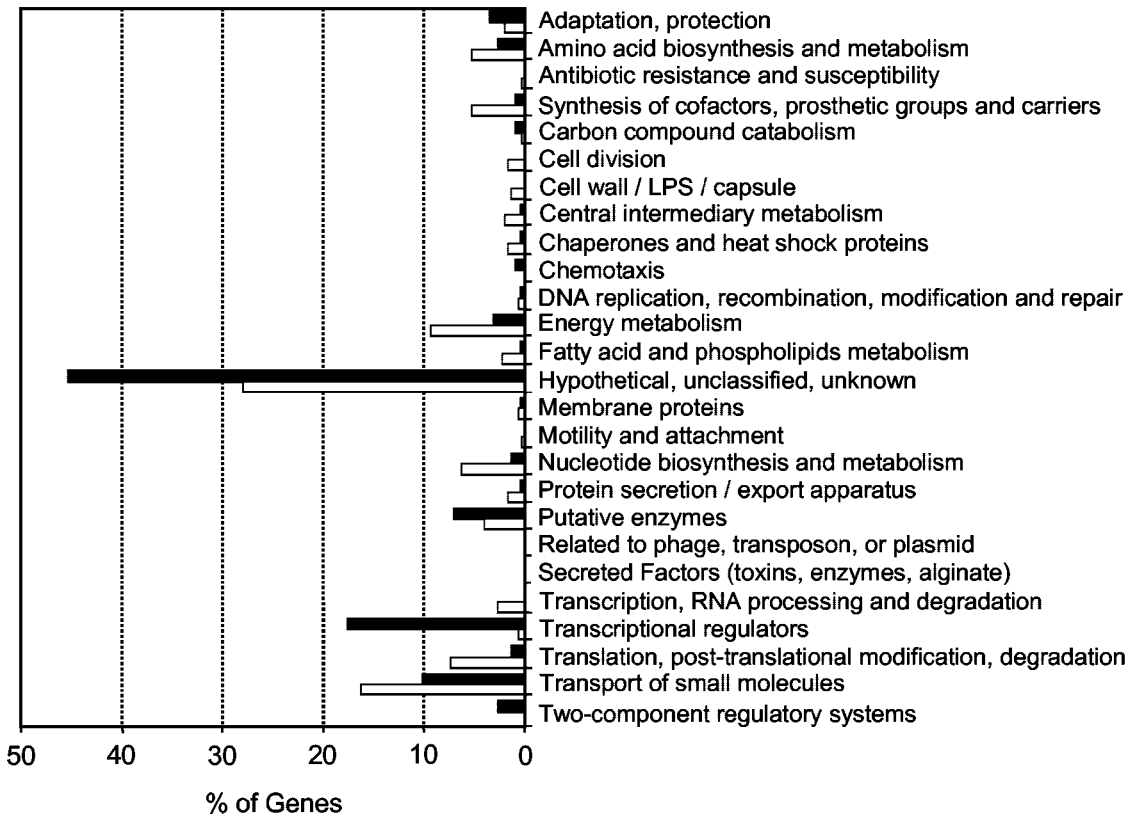

FIG. 1. Functional classification of genes with mRNA level changes of fivefold or more. The percentage of genes whose mRNA levels increased with treatment per functional class (closed bars) is relative to the total number of genes displaying ≥5-fold mRNA level increase with treatment. The percentage of genes whose mRNA levels decrease with treatment per functional class (open bars) is relative to the total number of genes displaying ≥5-fold mRNA level decrease with treatment. Functional classes are taken from http://www.pseudomonas.com/current_annotation.asp.

TABLE 2. List of the 40 genes whose mRNA levels displayed the highest fold changes with H_2O_2 treatment

Fold change	*P. aeruginosa* gene number (name)	Normalized gene signals[a]				Gene product description[b]
		Treated		Untreated		
		Mean*	*P* value	Mean	*P* value	
Increase						
218.0	3237	2.7660	0.0038	0.0127	0.0035	HP
120.5	4471 (*fagA*)	2.6260	0.0144	0.0218	0.0781	HP, *fur*-associated gene
105.1	4469	2.2770	0.0002	0.0217	0.0351	HP
80.0	3287	2.2990	0.0004	0.0287	0.0013	CHP
64.7	4470 (*fumC1*)	2.0950	0.0001	0.0324	0.0052	Fumarate hydratase
56.9	4613 (*katB*)	3.3220	0.0033	0.0584	0.0119	Catalase
53.0	4468 (*sodM*)	2.7030	0.0064	0.0510	0.1173	Superoxide dismutase
48.9	0250	2.2950	0.0004	0.0469	0.0038	CHP
45.3	1176 (*napF*)	3.1090	0.0543	0.0686	0.3505	Ferredoxin protein
45.0	4227 (*pchR*)	1.9880	0.0004	0.0442	0.2436	Transcriptional regulator
45.0	1230	1.7710	0.0230	0.0394	0.2376	HP
44.1	4221 (*fptA*)	7.7130	0.0191	0.1749	0.2192	Fe(III)-pyochelin outer membrane receptor
35.1	0472	1.8960	0.0002	0.0540	0.2908	Pr. sigma-70 factor, ECF subfamily
34.1	4236 (*katA*)	2.2470	0.0023	0.0659	0.0090	Catalase
29.4	4219	3.4170	0.0672	0.1162	0.1619	HP
27.2	2687 (*pfeS*)	2.3480	0.0063	0.0863	0.0499	Two-component sensor
26.7	1951	3.2280	0.0356	0.1209	0.0497	HP
26.0	3600	2.0860	0.0814	0.0802	0.0571	CHP
24.1	0672 (*hemO*)	1.9160	0.0007	0.0795	0.2338	Heme oxygenase
23.5	2288	2.0670	0.0015	0.0880	0.0085	HP
Decrease						
34.1	4131	0.1517	0.3044	5.1720	0.0300	Pr. iron-sulfur protein
25.8	2911	0.1128	0.0207	2.9100	0.0205	Pr. TonB-dependent receptor
24.3	0382 (*micA*)	0.0840	0.2686	2.0420	0.0027	DNA mismatch repair protein
21.7	0915	0.1118	0.1066	2.4270	0.0060	CHP
21.5	3608 (*potB*)	0.1233	0.1463	2.6500	0.0179	Polyamine transport protein
21.1	5074	0.1093	0.3200	2.3070	0.0100	Pr. ABC transporter component
19.6	5024	0.2462	0.0247	4.8250	0.0157	CHP
19.3	5479 (*gltP*)	0.1989	0.1328	3.8380	0.0208	Proton-glutamate symporter
19.2	4673	0.1662	0.0307	3.1910	0.0105	CHP
18.2	0889 (*aotQ*)	0.1610	0.0020	2.9300	0.0062	Arginine/ornithine transport protein
18.1	0605	0.2062	0.2255	3.7320	0.0298	Pr. permease of ABC transporter
16.3	4429	0.1450	0.2087	2.3640	0.0192	Pr. cytochrome c_1 precursor
15.4	0158	0.1045	0.3354	1.6090	0.0269	Pr. RND efflux transporter
15.3	4480 (*mreC*)	0.1305	0.0017	1.9960	0.0010	Rod shape-determining protein
14.4	0171	0.1514	0.1656	2.1800	0.0135	HP
13.9	4670 (*prs*)	0.1597	0.0354	2.2200	0.0031	Ribose-phosphate pyrophosphokinase
13.8	2444 (*glyA2*)	0.1254	0.0780	1.7300	0.0078	Serine hydroxymethyltransferase
13.4	4746	0.1701	0.0023	2.2790	0.0050	CHP
12.9	4055 (*ribC*)	0.1672	0.1985	2.1570	0.0057	Riboflavin synthase alpha chain
12.6	4672	0.1930	0.0005	2.4320	0.0055	Peptidyl-tRNA hydrolase

[a] Means of normalized gene signals of five replicates with corresponding *t* test *P* value.
[b] HP, hypothetical protein; CHP, conserved hypothetical protein; Pr., probable.

PA4471 (*fagA*, Fur-associated gene), *fumC* ($O_2^{\bullet -}$-resistant fumarase), PA4469 (unknown function), *sodM* (superoxide dismutase), and PA4467 (unknown function, located downstream of *sodM*). The PA4471-*fumC*-PA4469-*sodM* operon has been shown to be inducible by treatment with paraquat (27), Fur repressed, and inducible by iron starvation (6, 9, 10, 23, 24). Interestingly, a few additional iron starvation-inducible genes (*pchR*, *pchB*, *pchD*, *fptA*, *pvdS*, *pfeR*, *tonB*, PA0471, and PA0472) showed substantially increased mRNA levels (≥7- to 45-fold) in H_2O_2-treated cells. These results may suggest that H_2O_2-treated cells experience iron starvation and/or a transient loss of Fur repressor function (perhaps due to oxidative inactivation of Fur or oxidation and loss of the Fe^{2+} from the Fur-Fe^{2+} complex). The latter is more likely to be the reason for the increased transcript levels, since TS broth provides abundant iron.

Other genes that are relevant for oxidative stress adaptation and whose mRNA levels increased considerably (≥20-fold) were the catalase genes *katA* and *katB* and those of PA4612 (unknown function, adjacent to *katB*). Previous observations indicated that these genes are induced by H_2O_2 treatment; however, the reported induction of *katA* varied from a fewfold increase to undetectable change (3, 12, 14, 18, 22). The mRNA levels of PA2273 (*soxR*), encoding a possible sensor and transducer of oxidative stress and inducible by treatment with paraquat (8), showed a relatively modest but significant (threefold) increase. The mRNA levels of *ahpC*, *ahpF*, PA0848 (alkyl hydroperoxide reductases), *trxB2* (thioredoxin reductase), and

ohr (organic hydroperoxide resistance) also increased (4- to 22-fold). The *ahpCF* and *trxB*-PA0848 operons and *ohr* were recently shown to be induced by treatment with oxidant (21, 22). The mRNA levels of *gor* (glutathione reductase) increased fourfold, whereas the mRNA levels of the neighboring genes PA2826 (predicted glutathione peroxidase) and PA2827 (predicted transcriptional regulator) increased sevenfold or more. Notably, *hemH* mRNA increased fivefold. *hemH* encodes the ferrochelatase required for synthesis of heme, which is needed for synthesis of heme-containing peroxidases and catalases.

Overlap between the adaptive responses to H_2O_2 and agents that result in DNA damage was earlier documented in proteomic studies with *Enterobacteriaceae* (20). Consistent with these early observations, our studies revealed increases in mRNA levels of genes relevant for DNA repair or related functions in H_2O_2-treated cells. Most of these genes appear to be part of the *P. aeruginosa* SOS regulon, which was probably activated indirectly by H_2O_2 treatment due to oxidant-induced DNA damage. Among these genes are *recA* and *lexA*, whose mRNA levels increased 3- and 11-fold, respectively. DNA damage is known to induce the SOS regulon repressor *lexA* and the state of RecA that stimulates the autocatalytic cleavage of LexA to allow expression of the regulon (4, 17). Increased expression of *E. coli recA* and *lexA* or their gene products following cell treatment with DNA-damaging agents or H_2O_2 has been reported in previous studies (30–32). The mRNA levels of PA3008, adjacent to *lexA*, also increased (14-fold). The PA3008 product is similar to *E. coli* SulA/SfiA, a protein of the SOS regulon believed to arrest cell division until DNA repair processes are completed (16). The mRNA levels of PA0670 and PA0671 (in an apparent operon) increased 7- and 23-fold, respectively. The products of PA0670 and PA0671 are similar to DinP, a DNA damage-inducible protein of *Ralstonia solanacearum* (26) and *E. coli* SulA/SfiA, respectively. Also, mRNA levels of *recN* (DNA repair gene of the SOS regulon) and PA0962 (predicted DNA-binding stress protection protein) increased significantly (12- and 15-fold, respectively).

The fact that our analysis identified most genes previously known to be induced (directly or indirectly) by oxidants in *P. aeruginosa* validates the ability of our experiments to reveal candidate genes important for oxidative stress adaptation. Furthermore, to further validate our array methodology, real-time RT-PCR was used to determine relative mRNA levels of selected genes (e.g., PA3287, *potB*, *nuoA*, and *sodB*) in H_2O_2-treated *P. aeruginosa* compared with untreated controls. Increases or decreases determined by arrays and by RT-PCR in H_2O_2-treated cells were as follows: PA3287 mRNA increased 80-fold by array and 22- ($\pm$7)-fold by RT-PCR; *potB* mRNA decreased 21-fold by array and 28- ($\pm$6)-fold by RT-PCR; *nuoA* mRNA decreased 7-fold by array and 5- ($\pm$2)-fold by RT-PCR; and *sodB* mRNA levels were unchanged by array and by RT-PCR. Overall, the values obtained by RT-PCR have a good correspondence with the results of the arrays, taking into account the variation expected due to the different natures of the two methodologies.

Exposure to H_2O_2 also resulted in increased mRNA levels of virulence-related genes, such as the exoenzyme S and T genes, PA4937, and PA3239, with products similar to VacB and VacJ, respectively. VacB and VacJ are involved in virulence in *Enterobacteriaceae* (5, 29). Furthermore, transcript levels of *pvdS*, a gene required for exotoxin A and PrpL proteinase synthesis, increased in H_2O_2-treated cells. These observations could suggest that *P. aeruginosa* has a mechanism by which the oxidative environment in the host triggers an increase in the production of virulence factors, some of which might allow *P. aeruginosa* to avoid killing by incapacitating phagocyte functioning.

Genes whose mRNA levels decreased with H_2O_2 treatment. A large number of the genes with decreased mRNA levels in H_2O_2-treated cells are involved in primary metabolism (Table 2 and Supplemental Table 2). These genes are unlikely to be part of an oxidant-responsive regulon(s) and directly affected by H_2O_2 or part of the SOS regulon. Their mRNA level decreases probably reflect general changes in cell physiology and a transient metabolism slowdown as a consequence of sublethal oxidative damage resulting from treatment. For example, transcripts of genes involved in energy generation were markedly influenced; the mRNA levels of 5 of 13 *nuo* genes, encoding the NADH dehydrogenase complex I of the respiratory chain, decreased $\geq$5-fold, and the mRNA levels of 9 *atp* genes, encoding the F_1F_o ATP synthase complex of the oxidative phosphorylation, decreased $\geq$4-fold. Ribosomal biogenesis was also affected; e.g., the mRNA levels of 41 of 54 genes encoding 30S and 50S proteins (*rps*, *rpl*, *rpm*, and *prm* genes) decreased (16 of them $\geq$5-fold), while the remaining 13 genes showed unchanged mRNA levels. mRNA levels of genes from primary anabolic pathways such as nucleotide, fatty acid, and polyamine synthesis decreased as well. For example, (i) the mRNA levels of 11 of 21 purine and pyrimidine synthesis genes (*pur*, *gua*, and *pyr* genes) decreased $\geq$5-fold, whereas the rest remained unchanged or changed modestly; (ii) the mRNA levels of *accD*, *accC*, *acpP*, *fabF1*, *fabB*, and *fabA*, encoding enzymes of fatty acid synthesis, decreased 3- to 13-fold; and (iii) the mRNA levels of polyamine synthesis and uptake genes (*speA*, *speD*, *speE*, and *potABCD* operon) decreased $\geq$5-fold. Finally, the primary *sec*-dependent protein translocation pathway was also critically affected, with *secA*, *secB*, *secD*, *secE*, *secF*, *secG*, and *secY* mRNA levels decreasing 3- to 11-fold.

The presented work represents the first genome-wide investigation into the nature of the mRNA level changes induced in *P. aeruginosa* by exposure to a biologically relevant oxidant. Overall, the expression data indicate that the early response of *P. aeruginosa* to H_2O_2 consists of the following: (i) an upregulation of protective mechanisms, including production of cytotoxins that could impair immune cell functioning; and (ii) a downregulation of primary metabolism, perhaps influenced by growth arrest proteins encoded by PA0671 and PA3008 (with similarity to *E. coli* SulA and SfiA). Our results strengthen the confidence of previous assignments to the list of genes whose mRNA levels are modulated in response to an oxidant and add a significant number of *P. aeruginosa* genes likely to belong to this list. More importantly, our expression data provide clues as to the potential involvement of several genes listed as hypothetical, unclassified, and unknown in oxidative stress adaptation and constitute a genome-wide guide for mutagenesis analysis aimed at identifying novel functions important for the adaptation of *P. aeruginosa* to oxidative stress. Guided by our data, we have begun a mutagenesis analysis that has already indicated that PA0250 and PA3919 (genes listed as hypothetical, unclassified, and unknown and whose transcript levels increased with treatment) are required for optimal resistance

to H_2O_2. The way that these genes protect the cells against oxidants is currently under investigation.

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