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Microarray Analysis and Functional Characterization of the Nitrosative Stress Response in Nonmucoid and Mucoid *Pseudomonas aeruginosa*†

Aaron M. Firoved,[1] Simon R. Wood,[2,3] Wojciech Ornatowski,[1] Vojo Deretic,[1] and Graham S. Timmins[2]*

Department of Molecular Genetics and Microbiology[1] and College of Pharmacy, Toxicology Program,[2] University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131, and Division of Oral Biology, Leeds Dental Institute, University of Leeds, Leeds, United Kingdom[3]

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The type strain of *Pseudomonas aeruginosa*, PAO1, showed great upregulation of many nitrosative defense genes upon treatment with *S*-nitrosoglutathione, while the mucoid strain PAO578II showed no further upregulation above its constitutive upregulation of *nor* and *fhp*. NO· consumption however, showed that both strains mount functional, protein synthesis-dependent NO·-consumptive responses.

The prevalence of *Pseudomonas aeruginosa* in individuals with cystic fibrosis (CF) is thought to be due to derangements of salt concentrations in airway surface fluid, bacterial adhesion to airway epithelial cells, and nitric oxide (NO·)-mediated innate immunity (23). While these factors can be interrelated (5), decreased NO·-mediated innate immunity is clearly important (15). NO· is a potently bactericidal component of the innate immune system (3, 19) that acts either directly or via its ready conversion to other species, e.g., peroxynitrite and *S*-nitrosothiols. Microarray studies have demonstrated mucoidy-induced expression increases for genes whose products, such as nitric oxide reductase (*nor*) and flavohemoglobin (*fhp*), are involved in defense against NO· (7). Conversion to mucoidy in the CF-infected host increases general bacterial resistance to host clearance and antibiotics (11, 17), to which constitutive NO· defense by Nor and Fhp may contribute. To further characterize this NO· resistance, we induced nitrosative defense responses in the *P. aeruginosa* type strain, PAO1 (which is nonmucoid), and its mucoid derivative strain, PA0578II, by using *S*-nitrosoglutathione (GSNO). GSNO is a physiologically relevant NO· donor that provides a nonvolatile carrier of NO· in the airway surface fluid of the lung (27) and whose levels are decreased in the lower airways in individuals with CF (12). These characteristics are thought to be important in the pathogenesis of *P. aeruginosa* in cases of CF (10). Gene expression was determined by microarray analysis as previously described (7). Furthermore, rates of in vivo NO· consumption were measured by a microelectrode technique.

Microarray analysis of nitrosative defense by GSNO. GSNO (13) was added to cultures of PAO1 and its mucoid derivative, PAO578II (4, 8), for 30 min at a 5 mM final concentration, and then RNA extraction and analysis were performed as previously described (7). Strain PAO578II is a prototypical strain of mucoid isolates from individuals with CF: it carries both the *mucA22* and *sup-2* mutations (4, 8). The GSNO treatment caused growth arrest, but plate assays showed it not to be bactericidal (data not shown). The results from three microarray chips, i.e., independent identical experiments, were obtained for each strain (see Table S1 in the supplemental material). Each value from each chip represents the average of 13 independent spots for each gene on each chip, providing further averaging. Ratios of gene expression levels in GSNO-treated bacteria to those in controls were calculated, and the 30 most upregulated, annotated genes (28) for each strain were selected (see the online annotation project at http://www.pseudomonas.com). All statistical analysis was done by *t* testing (with Microcal Origin software).

The *P. aeruginosa* mucoid strain PAO578II and the nonmucoid strain PAO1 were grown and treated with GSNO as described above. Total cellular RNA was isolated by using the AquaPure RNA isolation kit (Bio-Rad) and treated with DNA-free (Ambion) to remove any contaminating DNA. Reverse transcription was performed with a Retroscript kit (Ambion) per the manufacturer's protocol. The total cDNA was quantified by spectrophotometry, and exactly 50 ng was used in each real-time PCR. Real-time PCR was carried out in triplicate on an iCycler iQ real-time PCR detection system (Bio-Rad) by using iQ SYBR Green Supermix (Bio-Rad) with 50 ng of cDNA and a 500 nM concentration of each primer. Controls consisted of samples to which no cDNA template had been added or to which original RNA was added. Primers were designed for *norB* and *fhp* with Primer Express software (Applied Biosystems, Foster City, Calif.). The PCR primers for *norB* were CCAATGGCTCCCTGAAATTC and GCCCGACGAAGAGGATCA. The primers for *fhp* were TGCGCCGCAACTATTCG and TTGACGCTGATGCGGTATTC. Following PCR, relative expression levels were calculated by using $2^{\Delta CT}$, where ΔCT represents the difference between cycling times (CT) for the two samples being compared. The CT is the point at which the PCR cycle crosses the preset logarithmic threshold.

Nonmucoid strain PAO1 strongly upregulates transcription of nitrosative defense genes upon GSNO treatment. Of the 30 most upregulated genes (upregulated more than threefold, with a *P* value of <0.05), 12 are involved with metabolizing

* Corresponding author. Mailing address: College of Pharmacy, Toxicology Program, University of New Mexico Health Sciences Center, Albuquerque, NM 87131. Phone: (505) 272-4103. Fax: (505) 272-6749. E-mail: gtimmins@salud.unm.edu.

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TABLE 1. Gene expression ratios for 30 most upregulated genes of *P. aeruginosa* PAO1 and corresponding genes of mucoid strain PAO578II under nitrosative stress with GSNO compared to controls

Gene	Gene expression ratio (with GSNO stress/control)[a]	
	PAO1	PAO578II
PA2664 *fhp*	38.7 (194)	1.72 (0.54)[b]
PA3877 *narK1*	27.9	9.31
PA3915 *moaB1*	22.9	25.4
PA0525 *norD*	19.6	0.87[b]
PA0523 *norC*	18.4	0.76[b]
PA0524 *norB*	18.4 (22.6)	0.88 (1.2)[b]
PA1671 *stk1*	6.97	0.96[b]
PA4225 *pchF*	5.79	6.62
PA1778 *cobA*	5.51	2.11[b]
PA0024 *hemF*	4.32	1.80
PA4235 *bfrA*	4.09	4.30
PA2532 *tpx*	3.88	2.58
PA3394 *nosF*	3.86	1.10[b]
PA0291 *oprE*	3.56	3.59
PA3392 *nosZ*	3.33	4.21
PA3746 *ffh*	3.19	4.01
PA4260 *rplB*	3.05	5.41
PA3549 *algJ*	3.04	0.24
PA1077 *flgB*	2.88	4.08
PA4687 *hitA*	2.82	1.52
PA3989 *holA*	2.77	4.87
PA1796 *folD*	2.74	2.91
PA3396 *nosL*	2.74	1.82
PA3391 *nosR*	2.69	1.19[b]
PA4267 *rpsG*	2.67	4.47
PA3393 *nosD*	2.57	1.04[b]
PA5563 *soj*	2.54	1.96
PA3246 *rluA*	2.50	1.83

[a] All results are significant at a P value of <0.05 except where otherwise indicated. Confirmatory real-time PCR was performed with *fhp* and *norB* as detailed in the text. Values in parentheses represent gene expression ratios as determined by PCR.

[b] Ratio indicates no significant difference in gene expression.

oxides of nitrogen and 2 have antioxidative functions (Table 1). The most highly upregulated gene, *fhp*, codes for flavohemoglobin, which oxidatively metabolizes $NO^{\cdot}$ to NO_3^- by using O_2 and NADPH (14) The genes *norB* and *norC* code for $NO^{\cdot}$ reductase (Nor), which reductively metabolizes $NO^{\cdot}$ to relatively inert N_2O and thus can protect against nitrosative stress. This parallel induction of both *nor* and *fhp* is consistent with the physiological need to detoxify $NO^{\cdot}$ as rapidly as possible. Nor, an integral inner membrane protein (32), is well placed to detoxify $NO^{\cdot}$ as it enters bacterial cells, while cytosolic Fhp can act only once $NO^{\cdot}$ has entered the cell. These capabilities can be viewed as providing nitrosative defense throughout the cell. To confirm the microarray analysis, real-time PCR was performed upon *fhp* and *norB*. In PAO1, upon GSNO treatment, the *fhp* and *norB* gene expression levels were 194- and 23-fold higher than those of the non-GSNO-treated controls, respectively ($P < 0.00001$). Other classes of genes involved in denitrification were also upregulated; their connections to the metabolic pathways are shown in Fig. 1. For example, *moaB1* codes for the synthesis of the molybdopterin cofactor of nitrate reductase, and *narK1* codes for a nitrate transporter (32). However, expression of *adhC*, glutathione-dependent formaldehyde dehydrogenase, which directly metabolizes GSNO and would be expected to be upregulated (16), was in fact not increased (0.8-fold increase; not significant).

The mucoid strain PAO578II displays limited upregulation of nitrosative defense genes but upregulates pyochelin and other stress proteins. The mucoid strain PAO578II exhibited a substantially different pattern of gene activation upon GSNO treatment, with a hallmark being little or no upregulation of the key nitrosative defense genes, *nor* and *fhp*, that are upregulated in PAO1 (Table 1). This finding was confirmed by real-time PCR analysis of *fhp* and *norB*. In the mucoid strain PAO578II, upon GSNO treatment the gene expression ratios for *fhp* and *norB* were increased 1.15-fold and decreased 1.9-fold, respectively, over those of the non-GSNO treated controls ($P < 0.025$). The *fhp* and *nor* genes are already greatly upregulated in mucoid cells; however, this is not the case for most of the other genes shown. The reason for this difference is unclear. The data showing little constitutive or GSNO-induced upregulation in mucoid strain PAO578II (compared to that in PAO1) of most genes (excluding *nor* and *fhp*) involved in denitrification are in contrast to the expectation that mucoid *P. aeruginosa* uses the denitrification pathway in respiration during mucoid colonization (30). One potential explanation, at least for the *nir* genes, is that Nir is proinflammatory due to increased epithelial-cell interleukin-8 production (18, 21). The lack of upregulation of *nir* is consistent with mucoidy-associated persistence in the lung and a decrease in the systemic virulence of most mucoid CF-associated isolates (31). The pat-

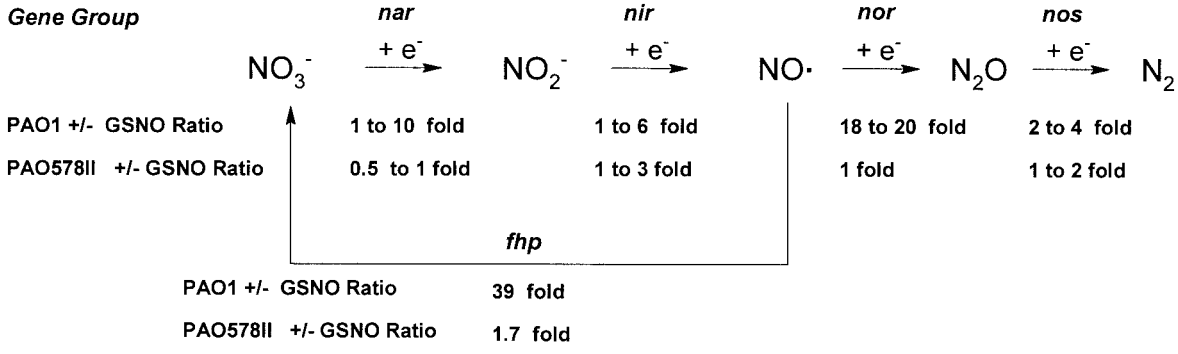

FIG. 1. Correlation between denitrification reaction pathway and increases in gene expression resulting from nitrosative stress, based on microarray data (see Table S1 in the supplemental material).

TABLE 2. Gene expression ratios for 30 most upregulated genes of *P. aeruginosa* PAO578II and corresponding genes of mucoid strain PAO1 under nitrosative stress with GSNO compared to controls

Gene	Gene expression ratio (with GSNO stress/control)[a]	
	PAO578II	PAO1
PA3915 *moaB1*	25.4	22.9
PA4231 *pchA*	19.0	8.4[b]
PA2850 *ohr*	13.7	6.3[b]
PA3407 *hasAp*	12.2	7.2[b]
PA4228 *pchD*	12.1	7.2[b]
PA4230 *pchB*	11.3	4.6[b]
PA4229 *pchC*	10.7	5.7[b]
PA0437 *codA*	10.0	1.4[b]
PA3877 *narK1*	9.31	27.9
PA4484 *gatB*	9.09	1.5[b]
PA3299 *fadD1*	8.87	2.1[b]
PA2629 *purB*	7.67	1.6[b]
PA1580 *gltA*	6.71	1.8[b]
PA4226 *pchE*	6.68	4.7[b]
PA4225 *pchF*	6.63	5.79
PA4266 *fusA1*	6.40	1.6[b]
PA5129 *grx*	6.10	2.4[b]
PA5128 *secB*	5.97	1.9[b]
PA4248 *rplF*	5.69	1.9[b]
PA5054 *hslU*	5.60	2.1[b]
PA4263 *rplC*	5.59	2.0[b]
PA0963 *aspS*	5.43	1.82
PA4260 *rplB*	5.41	2.7[b]
PA4762 *grpE*	5.38	2.1[b]
PA4246 *rpsE*	5.26	1.3[b]
PA4385 *groEL*	5.19	1.7[b]
PA4258 *rplV*	5.17	2.18
PA0447 *gcdH*	5.10	0.7[b]

[a] All results are significant at a P value of <0.05 except where otherwise indicated.
[b] Ratio indicates no significant difference in gene expression.

tern of upregulated genes in PAO578II (Table 2) was quite different from that of the genes in PAO1. One category of genes showing significant increases in expression in GSNO-treated mucoid strain PAO578II was that of the damage control and repair genes *bfr*, *groEL*, *grpE*, *grx*, *hslU*, and *ohr*, which were upregulated 4.3-, 5.2-, 5.4-, 6.1-, 5.6-, and 13.7-fold, respectively. These damage control and repair genes were not significantly upregulated in PAO1. Although *nor* and *fhp* are constitutively upregulated upon conversion to mucoidy (and hence in PAO578II) (7), this upregulation appears insufficient to completely protect against nitrosative damage; hence, these repair mechanisms are induced. Another major class of genes upregulated by GSNO in PAO578II was that of the *pch* genes that are involved in synthesis of the siderophore pyochelin (22, 24), which were also upregulated in PAO1 (although only the upregulation of *pchF* reached significance at a P value of <0.05). In particular, *pchA* (whose product is a rate-limiting step in pyochelin synthesis in *P. aeruginosa*) (9) is strongly upregulated. Expression of the pyochelin receptor gene (*fptA*) (1) was also increased 4.6-fold ($P < 0.05$). It is as yet unclear, however, whether this upregulation of *pchF* resulted from the known disregulation of iron metabolism caused by nitrosative stress (6) or from a metabolic requirement for iron. Pyoverdin genes were not upregulated significantly in PAO578II or PAO1 by GSNO.

TABLE 3. Comparison of gene expression ratios for *P. aeruginosa* PAO1 and mucoid strain PAO578II under nitrosative stress with GSNO with mucoidy-induced gene expression ratios determined previously for PAO578II

Gene	Gene expression ratio (with GSNO stress/control)[a]		
	PAO1	PAO578II[b]	Mucoidy-induced PAO578II[c]
PA0059 *osmC*	2.11[b]	0.71	24
PA0523 *norC*	18.4	0.76	52
PA0524 *norB*	18.4	0.88	54
PA0525 *norD*	19.6	0.87	20
PA0762 *algU*	0.85[b]	0.37	49
PA0763 *mucA*	1.57	0.58	21
PA1249 *aprA*	0.31[b]	0.50	9
PA1431 *rsaL*	1.81[b]	0.40	23
PA2193 *hcnA*	0.61[b]	1.05	6
PA2664 *fhp*	38.7	1.72	61
PA3540 *algD*	1.71[b]	0.52	7
PA3724 *lasB*	0.26[b]	1.39	8
PA4876 *osmE*	1.89[b]	0.68	49

[a] All results are significant at a P value of <0.05 except where otherwise indicated.
[b] Ratio(s) indicates no significant difference in gene expression.
[c] Data are from reference 7.

Gene expression responses of conversion to mucoidy in PAO578II and of nitrosative defense in PAO1 are essentially independent. A comparative study of the upregulation by nitrosative stress and mucoidy (shown for selected genes in Table 3) showed little cross-correlation between genes induced by mucoidy in PAO578II (7) and nitrosative stress. The control pathways involved in mucoidy and nitrosative defense appear to be essentially independent.

Expression of adherence genes is downregulated by nitrosative stress in both PAO1 and PAO578II. A recent study has shown that $NO^{\cdot}$ decreases adherence between *P. aeruginosa* and airway epithelial cells (5). Upon the onset of nitrosative stress, the expression of several important adherence genes, including *fliO* (26), *fliD* (2), and several *cupA* and *cupB* genes (29), was significantly downregulated in both PAO1 and mucoid strain PAO578II (Table 4). This downregulation may explain the decreased adherence, and maximizing this effect may provide a useful treatment strategy for CF.

In vivo $NO^{\cdot}$ consumption analysis. For the analysis of $NO^{\cdot}$ consumption, 45 min of GSNO exposure was used (to allow protein expression from increased mRNA), followed by cen-

TABLE 4. Gene expression ratios of potential adhesion-related genes in *P. aeruginosa* PAO1 and mucoid strain PAO578II under nitrosative stress with GSNO compared to control

Gene	Gene expression ratio (with GSNO stress/control)[a]	
	PAO1	PAO578II
PA1445 *fliO*	0.46	0.95[b]
PA2129 *cupA2*	1.35[b]	0.39
PA2132 *cupA5*	0.68[b]	0.30
PA4525 *pilA*	0.27	0.22
PA4082 *cupB5*	0.45[b]	0.33

[a] All results are significant at a P value of <0.05 except where otherwise indicated.
[b] Ratio indicates no significant difference in gene expression.

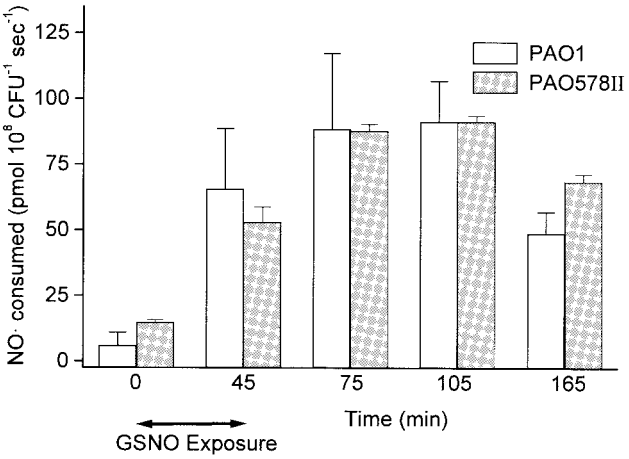

FIG. 2. NO· consumption by *P. aeruginosa* strains PAO1 and PAO578II (in the presence of 0.3 M NaCl) measured with an NO· electrode after nitrosative stress; data shown are means + 1 standard deviation ($n = 8$). Nitrosative stress consisted of exposure to GSNO (initial concentration, 5 mM) for 0 to 45 min, after which the bacteria were centrifuged and resuspended in GSNO-free LB medium. Consumption rates for PAO1 (except at time zero) were all statistically significant compared to those of either the same strain at time zero or the other strain at the same time point at a P value of <0.01 (t test).

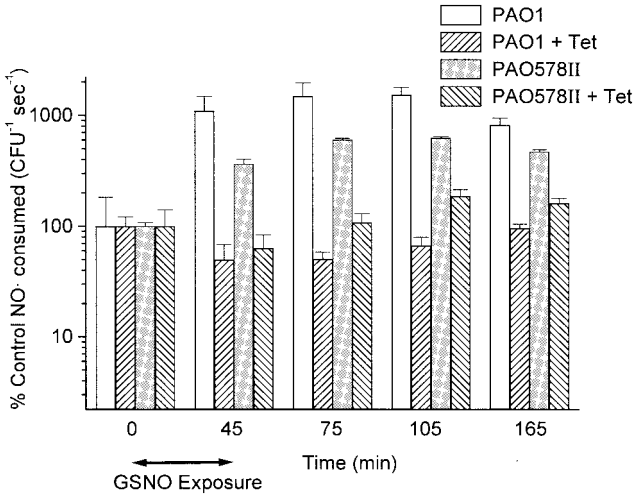

FIG. 3. Effect of protein synthesis inhibition by tetracycline (50 μg/ml) on NO· consumption by *P. aeruginosa* PAO1 and mucoid strain PAO578II as measured with an NO· electrode after nitrosative stress; data shown are means + 1 standard deviation ($n = 4$). Nitrosative stress consisted of exposure to GSNO (initial concentration, 5 mM) for 0 to 45 min, after which the bacteria were centrifuged and resuspended in GSNO-free LB medium still containing tetracycline. Consumption rates were analyzed as percentages of the initial GSNO-free rate, and all data points for strains PAO1 and PAO578II with or without tetracycline (except at time zero) were statistically significant at a P value of <0.01 (t test).

trifugation and resuspension in fresh Luria-Bertani (LB) medium. NO· (final concentration, 50 μM) was added to 1 ml of stirred aerobic culture (3×10^8 CFU/ml) in a glass chamber at 37°C (20). The NO· concentration was measured with a daily calibrated inNO-T system (Harvard Apparatus, Holliston, Mass.). The baseline NO· consumption of mucoid strain PAO578II (14.6 ± 1.1 pmol 10^8 CFU^{-1} s^{-1}) was significantly higher than that of nonmucoid strain PAO1 (5.8 ± 4.9 pmol 10^8 CFU^{-1} s^{-1}), at a P value of <0.01, in accordance with its higher expression of *nor* and *fhp* genes. NO· consumption was substantially increased with GSNO treatment for both PAO1 and PAO578II (Fig. 2). This GSNO-induced increase in NO· consumption was inhibited in both strains by the protein synthesis inhibitor tetracycline (Fig. 3). We observed a 15-fold increase in NO· consumption in PAO1 (from 0 to 105 min) (Fig. 2), consistent with the microarray data for *nor* and *fhp*. For mucoid strain PAO578II, the induction of NO· consumption by GSNO that we observed was sixfold higher than that demonstrated by the microarray data for PAO578II, in which neither *nor* nor *fhp* genes were upregulated. This difference may be the result of induction of an NO·-consuming system other than Nor or Fhp or of a posttranscriptional regulation process that increases synthesis of Nor or Fhp in the absence of increased mRNA.

These data could have implications for our understanding of *P. aeruginosa* pathogenesis in individuals with CF. For example, the limited effectiveness of clinical NO· or GNSO therapies for CF (25, 27) could derive in part from an induction of NO·-consuming, nitrosative defense systems, as demonstrated here. While this induction may explain the failure of these NO· and GSNO treatments, our studies with tetracycline suggest that combination therapy with a protein synthesis-inhibiting antibiotic could circumvent this problem. Additionally, characterization of NO· defenses of PAO1 and PAO578II at the protein level could provide an understanding of the nitrosative defense of clinically important mucoid strains.

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