The *Pseudomonas aeruginosa* Proteome during Anaerobic Growth‡

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Isotope-coded affinity tag analysis and two-dimensional gel electrophoresis followed by tandem mass spectrometry were used to identify *Pseudomonas aeruginosa* proteins expressed during anaerobic growth. Out of the 617 proteins identified, 158 were changed in abundance during anaerobic growth compared to during aerobic growth, including proteins whose increased expression was expected based on their role in anaerobic metabolism. These results form the basis for future analyses of alterations in bacterial protein content during growth in various environments, including the cystic fibrosis airway.

Pseudomonas aeruginosa is a ubiquitous environmental gram-negative bacterium found in soil and water. It is also an opportunistic pathogen that causes infections in individuals with innate immune defects, including cystic fibrosis (CF) patients (8). *P. aeruginosa* encounters low-oxygen environments in soil and water. Evidence indicates that in humans with CF, bacteria may, at least in part, be in a low-oxygen environment within mucopurulent masses or biofilms within the respiratory tracts (19). *P. aeruginosa* is able to grow anaerobically in the presence of terminal electron acceptors, such as nitrate (NO_3^-), nitrite (NO_2^-), and nitrous oxide (N_2O), or when L-arginine is a substrate for growth (21). The CF airway mucus is sufficiently rich in NO_3^- and NO_2^- to support the anaerobic growth of *P. aeruginosa* (7, 19). In this study, a comparison of the *P. aeruginosa* proteome during growth in the presence and absence of oxygen was performed.

P. aeruginosa strain PAO1 obtained from Steve Lory (Harvard Medical School, Boston, MA) was grown in 125-ml flasks in Luria broth (LB) supplemented with 1% KNO_3 with shaking at 200 rpm at 37°C for aerobic growth. Anaerobic growth was completed as previously described (9) in 80 ml of medium in 100-ml Wheaton serum bottles (Fisher Scientific) with rubber stoppers. The medium was deprived of oxygen by being subjected to bubbling with N_2 gas for 1 h. For both aerobic and anaerobic conditions, bacteria were harvested at the late logarithmic phase of growth, at which point the cell density (optical density at 600 nm) of the anaerobic culture was 44% of the density of the aerobic culture. There was no significant difference between the pHs of the harvested cultures (pH 7.6 for the anaerobic culture and pH 7.4 for the aerobic culture). Equal amounts of denatured and reduced whole-cell protein (2.0 mg from each growth state) were labeled with either light (^{12}C) or heavy (^{13}C) cleavable isotope-coded affinity tag

(ICAT) reagent (Applied Biosystems, Foster City, CA), processed, and analyzed as previously described (3). The reported data are the averages of at least two independent experiments.

Six hundred ten *P. aeruginosa* proteins were identified and quantified using ICAT (for a complete list of proteins, see Table S1 in the supplemental material). Among 151 proteins whose abundances changed during anaerobic growth, 76 were higher in abundance (Table 1) and 75 were lower in abundance (Table 2). As expected, 13 proteins that participate in anaerobic growth and denitrification (including products of *nir*, *nos*, and *nar* genes) were expressed at higher levels during anaerobic growth (Table 1). These results suggest that the observed changes in protein content include those resulting specifically from growth at different oxygen levels.

FIG. 1. *P. aeruginosa* secreted proteins expressed during anaerobic growth. *P. aeruginosa* culture supernatant proteins were separated by 12% SDS-PAGE and detected by staining with Coomassie. Proteins that changed in abundance during anaerobic growth (relative to aerobic growth) are labeled. $-\text{O}_2$, anaerobic growth; $+\text{O}_2$, aerobic growth.

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‡ Supplemental material for this article may be found at <http://jb.asm.org/>.

TABLE 1. *P. aeruginosa* proteins with increased abundance during anaerobic growth

Gene ^a	Protein	Gene name	<i>n</i> ^b	Ratio ^c	SD
PA0025*	Shikimate dehydrogenase	<i>aroE</i>	3	1.79	0.04
PA0130	Probable aldehyde dehydrogenase		10	2.28	0.22
PA0132	Beta-alanine-pyruvate transaminase		10	1.64	0.31
PA0286	Probable fatty acid desaturase		5	4.61	0.42
PA0300	Polyamine transport protein	<i>spuD</i>	7	1.65	0.17
PA0321	Probable acetylpolyamine aminohydrolase		1	1.91	NA ^d
PA0336	Nudix hydrolase YgdP	<i>ygdP</i>	13	1.54	0.40
PA0396	Twitching motility protein PilU	<i>pilU</i>	8	1.88	0.25
PA0408	Twitching motility protein PilG	<i>pilG</i>	2	1.63	0.10
PA0413	Component of signal transduction system	<i>chpA</i>	12	2.10	0.35
PA0520	Regulatory protein NirQ	<i>nirQ</i>	59	2.21	0.33
PA0655	Hypothetical protein		34	2.63	0.41
PA0658	Probable short-chain dehydrogenase		1	1.96	NA
PA0844	Hemolytic phospholipase C precursor	<i>plcH</i>	1	1.72	NA
PA0867	Hypothetical protein		4	2.33	0.12
PA0934	GTP pyrophosphokinase	<i>relA</i>	6	1.70	0.08
PA0936	LPS biosynthetic protein LpxO2	<i>lpxO2</i>	14	2.17	0.35
PA1155	Ribonucleoside reductase, small chain	<i>nrdB</i>	3	12.15	5.64
PA1156	Ribonucleoside reductase, large chain	<i>nrdA</i>	4	3.57	1.37
PA1398	Hypothetical protein		1	1.56	NA
PA1566	Conserved hypothetical protein		3	3.12	0.58
PA1681	Chorismate synthase	<i>aroC</i>	5	1.65	0.14
PA1766	Hypothetical protein		3	1.60	0.13
PA1847	Conserved hypothetical protein		1	1.88	NA
PA1919	Probable radical-activating enzyme		5	7.34	0.98
PA1920	Conserved hypothetical protein		15	10.80	5.21
PA2119	Alcohol dehydrogenase (Zn dependent)		25	1.84	0.22
PA2127	Conserved hypothetical protein		6	2.46	0.12
PA2323	Probable glyceraldehyde-3-phosphate dehydrogenase		2	1.95	NA
PA2567	Hypothetical protein		1	1.54	NA
PA2945	Conserved hypothetical protein		2	2.36	0.30
PA2991	Soluble pyridine nucleotide transhydrogenase	<i>sth</i>	20	1.93	0.37
PA2994	NA ⁺ -translocating NADH:quinone oxidoreductase	<i>nqrF</i>	15	1.60	0.27
PA2999*	NA ⁺ -translocating NADH:ubiquinone oxidoreductase	<i>nqrA</i>	5	1.74	0.11
PA3002	Transcription-repair coupling protein Mfd	<i>mfd</i>	2	1.52	0.06
PA3150	LPS biosynthesis protein WbpG	<i>wbpG</i>	1	3.72	NA
PA3185	Hypothetical protein		4	1.82	0.08
PA3391	Regulatory protein NosR	<i>nosR</i>	5	7.75	0.98
PA3392	Nitrous oxide reductase precursor	<i>nosZ</i>	69	3.65	0.72
PA3394	NosF protein	<i>nosF</i>	9	4.09	0.46
PA3438	GTP cyclohydrolase I precursor	<i>folEI</i>	1	5.58	NA
PA3515	Hypothetical protein		1	4.21	NA
PA3562*	Probable phosphotransferase system enzyme I		3	2.91	0.17
PA3694	Hypothetical protein		4	1.92	0.07
PA3871	Probable peptidyl-prolyl <i>cis-trans</i> isomerase, PpiC type		3	2.50	0.64
PA3873	Respiratory nitrate reductase delta chain	<i>narJ</i>	1	3.20	NA
PA3874	Respiratory nitrate reductase beta chain	<i>narH</i>	67	7.89	2.83
PA3875	Respiratory nitrate reductase alpha chain	<i>narG</i>	35	7.70	3.23
PA3880	Conserved hypothetical protein		8	3.88	0.98
PA3886	Hypothetical protein		1	7.25	NA
PA3895	Probable transcriptional regulator		2	1.49	0.00
PA3913	Probable protease		1	5.30	NA
PA3914*	Molybdenum cofactor biosynthetic protein A1	<i>moeA1</i>	21	3.41	0.63
PA3915*	Molybdopterin biosynthetic protein B1	<i>moaB1</i>	5	4.40	0.72
PA3918*	Molybdopterin biosynthetic protein C	<i>moaC</i>	23	1.88	0.41
PA3958	Hypothetical protein		1	2.29	NA
PA4180	Probable acetolactate synthase large subunit		2	2.16	0.53
PA4811	Nitrate-inducible formate dehydrogenase, beta subunit	<i>fdnH</i>	3	5.85	1.85
PA4812	Formate dehydrogenase-O, major subunit	<i>fdnG</i>	4	3.46	0.64
PA4868	Urease alpha subunit	<i>ureC</i>	1	1.51	NA
PA4922	Azurin precursor	<i>azu</i>	4	2.96	0.81
PA5005	Probable carbamoyl transferase		42	1.59	0.24
PA5011	Heptosyltransferase I	<i>waaC</i>	4	1.49	0.17
PA5012	Heptosyltransferase II	<i>waaF</i>	6	1.45	0.11
PA5015	Pyruvate dehydrogenase	<i>aceE</i>	111	1.98	0.42
PA5064	Hypothetical protein		1	1.93	NA
PA5223	UbiH protein	<i>ubiH</i>	3	1.67	0.10
PA5296	ATP-dependent DNA helicase Rep	<i>rep</i>	2	1.77	0.00

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TABLE 1—Continued

Gene ^a	Protein	Gene name	n ^b	Ratio ^c	SD
PA5300	Cytochrome <i>c</i> ₅	<i>cycB</i>	13	1.91	0.21
PA5332	Catabolite repression control protein	<i>crc</i>	3	1.90	0.21
PA5440	Probable peptidase		1	18.54	NA
PA5496*	Hypothetical protein		8	6.46	2.07
PA5497*	Hypothetical protein		10	11.28	3.17
PA5508	Probable glutamine synthetase		11	2.73	0.26
PA5564	Glucose inhibited division protein B	<i>gidB</i>	2	1.53	0.02

^a Genes marked with an asterisk (*) were identified as up-regulated during anaerobic growth (1).
^b Number of peptides identified and quantified for each protein.
^c Values represent relative protein abundance, or the ratio of protein expression in cells grown anaerobically to protein expression in cells grown aerobically.
^d NA, not applicable.

The changes in the detected proteome could also reflect differences in all density-dependent regulation in addition to effects of oxygen tension, given the lower relative cell density of the harvested anaerobic culture. Indeed, 29 proteins detected in lower abundance in anaerobically grown cells are encoded by genes previously shown to be quorum sensing induced (5, 16, 17). These include the hydrogen cyanide synthase subunits HcnB and HcnC; the *Pseudomonas* quinolone signal biosynthetic enzymes PqsB, PqsC, and PqsD; and PhnB (Table 2). Consistent with our results, *hcn* and *pqs* genes were also found to be transcriptionally repressed during anaerobic growth by a

recent DNA microarray analysis using aerobic and anaerobic cultures harvested at the same cell density (1) (Table 2). To identify secreted *P. aeruginosa* proteins with altered levels during anaerobic growth, culture supernatant proteins were concentrated (11) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Four Coomassie-stained protein bands, corresponding to differentially expressed proteins, were identified and analyzed as in a previous study (4) (Fig. 1). The abundances of three secreted proteins appeared to decrease during anaerobic growth: the CbpD chitin-binding protein, LasB elastase, and a protein of

FIG. 2. *P. aeruginosa* outer membrane proteins expressed during anaerobic growth. Outer membrane proteins were separated by 12% 2D PAGE and detected by staining with Coomassie. Proteins were separated in the first dimension by isoelectric focusing (IEF) at pI ranges of 4 to 7 (A) and 6 to 11 (B). Proteins that changed in abundance during anaerobic growth (relative to aerobic growth) are labeled with arrows. -O₂, anaerobic growth; +O₂, aerobic growth.

TABLE 2. *P. aeruginosa* proteins with decreased abundance during anaerobic growth

Gene ^a	Protein ^c	Gene name	n ^b	Ratio ^c	SD
PA0085	Conserved hypothetical protein		3	2.15	0.26
PA0100	Hypothetical protein		1	1.53	NA ^d
PA0128	Conserved hypothetical protein		9	2.10	0.35
PA0139	Alkyl hydroperoxide reductase subunit C	<i>ahpC</i>	655	2.50	1.29
PA0195	Still frameshift pyridine nucleotide transhydrogenase	<i>pntA</i>	10	2.21	0.55
PA0399	Cystathionine beta-synthase		6	3.39	0.52
PA0447*	Glutaryl-CoA dehydrogenase	<i>gcdH</i>	24	5.30	1.04
PA0534	Conserved hypothetical protein		4	5.46	1.51
PA0588	Conserved hypothetical protein		78	5.56	2.52
PA0746	Probable acyl-CoA dehydrogenase		2	2.52	0.51
PA0853	Probable oxidoreductase		16	2.19	0.30
PA0854	Fumarate hydratase	<i>fumC2</i>	9	2.36	0.34
PA0870	Aromatic amino acid aminotransferase	<i>phhC</i>	24	1.74	0.22
PA0871	Pterin-4-alpha-carbinolamine dehydratase	<i>phhB</i>	27	2.37	0.57
PA0872	Phenylalanine-4-hydroxylase	<i>phhA</i>	60	2.11	0.65
PA0916	Conserved hypothetical protein		6	1.93	0.28
PA0997*	Quinolone signal biosynthesis protein	<i>pqsB</i>	3	15.54	6.73
PA0998*	Quinolone signal biosynthesis protein	<i>pqsC</i>	5	9.11	3.39
PA0999*	3-Oxoacyl-[acyl-carrier-protein] synthase III	<i>pqsD</i>	12	5.62	1.50
PA1002*	Anthranilate synthase component II	<i>phnB</i>	1	2.30	NA
PA1228	Hypothetical protein		13	2.55	0.52
PA1529	DNA ligase	<i>lig</i>	21	2.50	0.33
PA1574	Conserved hypothetical protein		1	2.25	NA
PA1662	Probable ClpA/B-type protease		2	2.65	0.27
PA1756	3'-Phosphoadenosine-5'-phosphosulfate reductase	<i>cysH</i>	3	2.89	0.13
PA1772	Probable methyltransferase		4	2.34	0.43
PA1894	Hypothetical protein		9	2.48	0.94
PA1964	Probable ATP-binding component of ABC transporter		1	1.00	NA
PA2001	Acetyl-CoA acetyltransferase	<i>atoB</i>	149	1.74	1.11
PA2007	Maleylacetoacetate isomerase	<i>maiA</i>	10	2.45	0.47
PA2008	Fumarylacetoacetase	<i>fahA</i>	47	11.02	4.75
PA2009	Homogentisate 1,2-dioxygenase	<i>hmgA</i>	4	20.39	11.50
PA2012*	Probable acyl-CoA carboxylase alpha chain		7	2.24	0.19
PA2014*	Probable acyl-CoA carboxyltransferase beta chain		69	2.14	0.44
PA2044	Hypothetical protein		4	3.49	0.24
PA2069	Probable carbamoyl transferase		10	4.11	1.13
PA2081	Hypothetical protein		4	2.25	0.12
PA2112*	Conserved hypothetical protein		28	3.94	0.90
PA2116	Conserved hypothetical protein		35	3.67	0.97
PA2194	Hydrogen cyanide synthase HcnB	<i>hcnB</i>	9	3.26	0.50
PA2195	Hydrogen cyanide synthase HcnC	<i>hcnC</i>	3	4.11	0.15
PA2247	2-Oxoisovalerate dehydrogenase (alpha subunit)	<i>bkdA1</i>	7	3.54	1.00
PA2248	2-Oxoisovalerate dehydrogenase (beta subunit)	<i>bkdA2</i>	59	2.80	1.07
PA2250	Lipoamide dehydrogenase Val	<i>lpdV</i>	18	2.79	0.59
PA2366*	Conserved hypothetical protein		1	2.70	NA
PA2552*	Probable acyl-CoA dehydrogenase		13	1.99	0.70
PA2553*	Probable acyl-CoA thiolase		48	2.17	0.50
PA2555*	Probable AMP-binding enzyme		10	2.22	0.56
PA2850	Organic hydroperoxide resistance protein	<i>ohr</i>	6	2.26	0.37
PA2939	Probable aminopeptidase		3	2.67	0.80
PA2981	Tetraacyldisaccharide 4'-kinase	<i>lpxK</i>	1	13.49	NA
PA3049	Ribosome modulation factor	<i>rmf</i>	15	3.84	0.92
PA3195	Glyceraldehyde 3-phosphate dehydrogenase	<i>gapA</i>	1	2.76	NA
PA3327	Probable nonribosomal peptide synthetase		1	2.16	NA
PA3328	Probable FAD-dependent monooxygenase		6	4.58	1.28
PA3329*	Hypothetical protein		1	2.08	NA
PA3331	Cytochrome P450		17	5.10	2.00
PA3347	Hypothetical protein		4	1.96	0.20
PA3365	Probable chaperone		1	2.35	NA
PA3366	Aliphatic amidase	<i>amiE</i>	1	2.00	NA
PA3481	Conserved hypothetical protein		1	1.54	NA
PA3537	Ornithine carbamoyltransferase, anabolic	<i>argF</i>	1	5.57	NA
PA3569	3-Hydroxyisobutyrate dehydrogenase	<i>mmsB</i>	25	3.67	0.94
PA3570	Methylmalonate-semialdehyde dehydrogenase	<i>mmsA</i>	1	3.17	NA
PA3842	Probable chaperone		8	3.17	1.44
PA3919*	Conserved hypothetical protein		7	2.19	0.36
PA4015	Conserved hypothetical protein		11	2.11	0.67
PA4129*	Hypothetical protein		3	3.75	1.17

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TABLE 2—Continued

Gene ^a	Protein ^c	Gene name	n ^b	Ratio ^c	SD
PA4132	Conserved hypothetical protein		6	2.36	1.11
PA4217	Flavin-containing monooxygenase	<i>phzS</i>	5	5.09	1.26
PA4362	Hypothetical protein		2	2.17	0.29
PA4412*	MurG protein	<i>murG</i>	1	3.28	NA
PA4498	Probable metalloproteinase		4	2.00	0.06
PA5100	Urocanase	<i>hutU</i>	10	4.50	1.23
PA5410	Probable ring hydroxylating dioxygenase, alpha subunit		1	2.76	NA

^a Genes marked with an asterisk (*) were identified as down-regulated during anaerobic growth (1).

^b Number of peptides identified and quantified for each protein.

^c Values represent relative protein abundance, or the ratio of protein expression in cells grown aerobically to protein expression in cells grown anaerobically.

^d NA, not applicable.

^e CoA, coenzyme A; FAD, flavin adenine dinucleotide.

unknown function encoded by PA0572. Previous proteomic studies found that all three of these proteins are quorum sensing induced (11). One protein appeared to be increased in abundance during anaerobic growth and was identified as either the flagellar filament protein FliC or the flagellar capping protein FliD (due to the overlap in characteristics of these two proteins).

Most *P. aeruginosa* outer membrane proteins do not contain cysteine residues and hence cannot be analyzed by ICAT (4). Therefore, two-dimensional (2D) PAGE was used as a complementary method (4). Several outer membrane proteins (Fig. 2) were excised from the 2D gel and identified (4). OprE appeared to increase in abundance during anaerobic growth, while OprF and OprH appeared to decrease in abundance (Fig. 2). All three proteins migrated as multiple species during isoelectric focusing (Fig. 2). Decreased abundance of OprF during anaerobic growth was confirmed by immunoblotting of outer membrane proteins (data not shown), using a polyclonal anti-OprF antiserum (a gift from Robert Hancock, University of British Columbia at Vancouver, Canada).

Among the *P. aeruginosa* proteins that showed increased abundance during anaerobic growth (Table 1; Fig. 1), several contribute to functions involved in the formation and development of biofilms. These proteins include the catabolite repression control protein Crc and the twitching motility proteins PilU, PilG, and ChpA (12, 13, 18). Consistent with an increased level of Crc in anaerobically grown cells (Table 1), known targets of Crc repression were decreased in abundance (Table 2), including the *hmgA* and *bkd* gene products (6, 10). ChpA and PilG are components of a complex regulatory system controlling twitching motility (18). Taken together, these results suggest that expression or function of cell surface appendages that affect biofilm formation is altered during anaerobic growth. Such changes may contribute to the increased biofilm formation observed for *P. aeruginosa* growing anaerobically (20).

In addition to the changes in outer membrane proteins observed during anaerobic growth, ICAT analysis showed that several enzymes involved in the biosynthesis of *P. aeruginosa* lipopolysaccharide (LPS) were expressed at higher levels during anaerobic growth (Table 1). These included a homologue of beta-hydroxylase LpxO2, which hydroxylates lipid A fatty acids (14); LPS core heptosyltransferases WaaC and WaaF (2, 15); and WbpG, which is encoded by a gene cluster that participates in the synthesis of a long B-band O antigen. These

results suggest that LPS content could be altered as a consequence of anaerobiosis.

In summary, the *P. aeruginosa* proteome changes significantly during anaerobic growth. We identified 617 proteins in total: 610 by ICAT analysis, 4 by SDS-PAGE analysis, and 3 by 2D PAGE analysis. Of the 617 identified proteins, the abundances of 158 varied between anaerobically grown and aerobically grown cells. Because *P. aeruginosa* reached a lower cell density under our anaerobic growth conditions than under aerobic growth conditions, density-dependent changes in protein expression may have contributed to the proteome that we detected during anaerobic growth. Nevertheless, bacterial cell density is likely to be similarly limited in many environmental niches where multiple nutrients (including oxygen) are scarce. Therefore, the changes in protein levels that we have detected contribute to an understanding of how the proteome and metabolic state of bacteria vary in response to different environments. Direct analysis of bacterial protein content is a robust technology to observe the adaptation of bacteria to specific environmental niches, including the CF airway.

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