Genetically susceptible bacteria become antibiotic tolerant during chronic infections, and the mechanisms responsible are poorly understood. One factor that may contribute to differential sensitivity in vitro and in vivo is differences in the time-dependent tobramycin concentration profile experienced by the bacteria. Here, we examine the proteome response induced by subinhibitory concentrations of tobramycin in Pseudomonas aeruginosa cells grown under planktonic conditions. These efforts revealed increased levels of heat shock proteins and proteases were present at higher dosage treatments (0.5 and 1 μg/ml), while less dramatic at 0.1 μg/ml dosage. In contrast, many metabolic enzymes were significantly induced by lower dosages (0.1 and 0.5 μg/ml) but not at 1 μg/ml dosage. Time course proteome analysis further revealed that the increase of heat shock proteins and proteases was most rapid from 15 min to 60 min, and the increased levels sustained till 6 h (last time point tested). Heat shock protein IbpA exhibited the greatest induction by tobramycin, up to 90-fold. Nevertheless, deletion of ibpA did not enhance sensitivity to tobramycin. It seemed possible that the absence of sensitization could be due to redundant functioning of IbpA with other proteins that protect cells from tobramycin. Indeed, inactivation of two heat shock chaperones/proteases in addition to IbpA with other proteins that protect cells from tobramycin, even when the infecting bacteria are antibiotic sensitive, as is the case early in disease.

The opportunistic pathogen Pseudomonas aeruginosa is ubiquitous in the natural environment and causes human infections (1). P. aeruginosa can metabolize various carbon and nitrogen compounds and persists under nutrient-poor and hostile growth environments (2, 3). One example is P. aeruginosa pulmonary infection of cystic fibrosis (CF) patients. Despite stress induced by host defenses and high concentrations of antibiotics, P. aeruginosa cells are able to persistently colonize CF airways (4).

The aminoglycoside tobramycin is a front-line drug currently used in the treatment of P. aeruginosa in CF and other diseases. It is supplied in the forms of inhaled solution (TOBI) and intravenous injection. The tobramycin concentrations in airways after 300-mg dosage TOBI inhalation can reach 1,000 μg per g of sputum (5, 6). This concentration is in the range of 10 to 1,000 times of the minimal inhibitory concentration (MIC) for P. aeruginosa clinical isolates tested ex vivo (6). However, even with such high tobramycin concentrations, chronic P. aeruginosa infections are rarely eradicated (6). This is true even when the infecting bacteria are antibiotic sensitive, as is the case early in disease.

One possible reason for P. aeruginosa persistence in vivo could relate to the time dependence of local concentrations of tobramycin experienced by P. aeruginosa in CF patient airways. Many factors, including inflammatory responses, blood and lymphatic circulations, and air flow distribution (for inhaled antibiotics), can alter the local antibiotic concentrations. In addition, P. aeruginosa cells can form biofilms in CF lungs and other infection sites (8), and biofilm exopolysaccharide layers may slow the diffusion of tobramycin (9, 10). P. aeruginosa cells in the inner layers of biofilms may experience lower concentrations and more gradual increase of tobramycin levels than those in outer layers (10, 11). Furthermore, even if final tobramycin concentration levels inside the biofilm eventually grow to match the highest levels experienced elsewhere, bacteria in these inner regions have experienced a
slower increase, during which time proteome levels could be altered to promote the “adapted resistant state” (12). Adaptive resistance can also be induced in planktonic (free-living) P. aeruginosa (13, 14), and conventional MIC assays are not designed to measure this.

Once induced, the adaptive resistance confers bacteria higher resistance to antibiotic treatments (13, 14) and is associated with decreased clinical antibiotic treatment efficacy (15). Interestingly, the adaptive resistance is time dependent and reversible. Typical adaptive resistance was observed starting 1 h after antibiotic exposure, and the drug susceptibility was regained after 36 h intervals (14, 15). Thus, adaptive resistance mechanisms may contribute in part to the disparity of in vivo persistence and ex vivo susceptibility to antibiotics in MIC tests.

As an initial step toward defining adaptive resistance mechanisms, we investigated the time- and concentration-dependence of P. aeruginosa proteome response to tobramycin in planktonic conditions. Since the most effective protective responses may operate before killing begins and the rate of change of drug levels is likely to depend on ambient conditions, we studied bacteria exposed to low, subinhibitory levels of tobramycin (0.1, 0.5, and 1.0 μg/ml) at a range of time points (15, 60, 120, and 360 min) after exposure. The candidate proteome marker of P. aeruginosa for tobramycin response, heat shock protein IbpA, was further investigated with genetic mutagenesis and MIC assays.

**EXPERIMENTAL PROCEDURES**

**P. aeruginosa Strains—**P. aeruginosa strain MPAO1 (16) was used for proteome analysis. Single transposon insertion mutants were obtained from the two-allele mutant library (17). The specific mutants examined were as follows: PA0779 (PW2411, PW2413, PW2414), clpB (PW8651, PW8652), hslV (PW9485, PW9486, PW9487), and the transposon-containing control PA3303 (18).

Two ibpA loss-of-function mutant alleles were used in this study. The first ibpA::luxCDABE was constructed in two recombination steps by replacing wild-type ibpA with an ibpA::lux gene fusion carried on plasmid pEX19Tc (19). The lux insertion site was at the N-terminal fifth amino acid of ibpA. The structure of the constructed allele was confirmed by PCR, and the absence of Ibpa expression was confirmed by the selected reaction monitoring (SRM)1. The lux control strain PKH181 was constructed by insertion of pUC18-mini-Tn7T-Gm-lux vector (without a cloned promoter) into MPA01, according to methods of Choi and Schweizer (20). The second ibpA mutant (ΔibpA) corresponded to an in-frame deletion that joined sequences coding for the N-terminal fifth amino acid to the fourth amino acid from the C terminus. The deletion was generated by recombination in MPA01 with pEX19Tc plasmid carrying the deletion allele. The in-frame ΔftsH deletion mutant has been previously described (21).

To generate the double mutants (ibpA/P A0779, ibpA/clpB, and ibpA/hslV), chromosomal DNA isolated from the transposon-containing strains was transformed into ibpA::lux insertion inactivation mutant via lambda red recombination. Positive transformants were selected by tetracycline resistance as previously described (22).

**Tobramycin Treatment for Proteomics Samples—**MPAO1 cells were grown in salt-free LB containing 10 g tryptone, 5 g yeast extract per liter at 37 °C to optical density OD_{600} = 1.0, and aliquotted to 5 ml volumes. For the dosage-dependent treatments, tobramycin (Sigma-Aldrich, St. Louis, MO) at 0.1, 0.5, or 1.0 μg/ml (final concentration) was added to the cell cultures with shaking for 60 min at 37 °C. For the time course treatments, cells were treated with 1.0 μg/ml tobramycin (final concentration) were exposed for 15, 60, 120, and 360 min at 37 °C. Cells harvested at time zero (at the time that tobramycin treatment started) were used as controls. Three biological replicates for dosage treatments and two biological replicates for the time course were analyzed.

**Tobramycin MIC Assays—**Minimum inhibitory concentration (MIC) assays were performed as described by Lee et al. (18). Single colonies were inoculated into 96-well plates containing 250 μl salt-free LB per well and grown for 18 h at 37 °C in a humidity chamber. 200 μl of these cultures were removed, added to a new plate, and 10-fold dilutions were made with salt-free LB. The salt was not added in LB for initial liquid culture because ΔibpA mutant is salt sensitive (21), and salt-free conditions resulted in relatively uniform growth for strains. Diluted cells were allowed to grow for 90 min at 37 °C in a humidity chamber, at which point cells were spotted to tobramycin LB agar (including 137 mM NaCl) to deposit approximate 10^5 cells per spot. Plates were incubated at 37 °C for 18 h and photographed. MIC was defined as the lowest antibiotic concentration preventing the lawn growth of the spotted cells.

**Protein Extraction of P. aeruginosa Cells—**MPAO1 cells were harvested by centrifugation at 3,500 rpm for 10 min. Cell pellets were resuspended with lysis buffer (50 mM ammonium bicarbonate, pH 8.0; 8 M urea; and 10 mM DTT). Cells were lysed by incubation in lysis buffer on ice for 30 min, with vortexing for 30 s every 5 min. Cells in lysis buffer underwent one –80 °C freeze-thaw cycle to maximize cells lysis. Protein extracts were briefly sonicated to shear DNA. Cell debris was removed by centrifugation at 20,000 g for 20 min. Extracted protein concentration was measured with Bradford assay (Thermo Fisher Scientific, Waltham, MA).

100 μg proteins from each extraction were diluted twofold with digestion buffer (40 mM ammonium bicarbonate, 5% acetonitrile) for alkylolation by 10 mM iodoacetamide for 30 min in the dark. The mixture then was further diluted to urea concentration less than 1.5 M with digestion buffer for overnight trypsin digestion (Promega, Madison, WI). Peptides were purified with Sep-Pak cartridges (Waters Corporation, Milford, MA), dried, and resuspended with 0.1% formic acid. 1 μg of tryptic digest was loaded in each LC-MS/MS analysis.

**LC-MS/MS Analysis—**The proteomics samples were analyzed with NanoAcquity UPLC (Waters) coupled to a linear quadrupole ion trap mass spectrometer (LTQ-XL, from Thermo Fisher Scientific). The column length of 3 cm trap column packed in house with 200 Å C18 magic beads (Bruker-Michrom Inc, Auburn, CA) and 30 cm analytical column packed with 100 Å C18 magic beads were used in all LC/ MS-MS analysis to maintain the uniformity of peptide chromatography profiles. A 120 min linear gradient (5–35% acetonitrile) at a flow rate of 300 nl/min was used, and peptides were ionized by electrospray.

Data-dependent acquisition (DDA) mode was used in the spectral counting analysis. The top five most intense ions in the MS1 scan were selected for tandem mass spectrometry analysis. For the selected reaction monitoring (SRM) analysis, scan-type SRM was used. Targeted peptides and transition ions were manually selected from DDA identification spectra. Ion intensity and preferentially larger fragment masses were the main selection criteria. Five to eight fragment ions were included per peptide. SRM instrument set-
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For this study, all proteins were standardized, which included parent ions isolation width 3 m/z, transition ions scan width of 1 m/z, collision-induced disassociation (CID) collision energy 35, activation Q 0.25, activation time 30 ms, and automatic gain control (AGC) MSn value 1 × 104, maximum ion time 500 ms.

Data Analysis—The DDA raw files were converted to mgf peak list files using ReAdW (Ver. 4.3.1) (23). The data were searched in Mascot (version 2.3.02) against the Pseudomonas aeruginosa (PA01) database (consisting of 5,680 genes or splicing fragments, annotation version 2012-Nov) (24). The search parameters included 3.0 Da precursor mass tolerance, 0.6 Da fragment mass tolerance, fixed modifications cysteine carbamidomethylation, variable modification methionine oxidation, digestion enzyme trypsin, and maximum missed cleavages 2. The peptide expectation value 0.05 was used as the cutoff, and the false discovery rate (FDR) was determined by search-
cleavages 2. The peptide expectation value 0.05 was used as the
cutoff, and the false discovery rate (FDR) was determined by search-

The Exponentially Modified Protein Abundance Index (emPAI) values derived from Mascot search were used for spectral quantitation analysis (25). The data of a total of seven LC-MS/MS analyses for each dosage treatment and five LC-MS/MS analyses for each time course treatment were used for spectral counting statistical analysis. We required proteins for quantitation analysis to be quantified minimally by three unique peptides from at least two biological replicates. Proteins with significant fold changes (P ≤ 0.05) as well as proteins with no detection in one state but detected more than four times in another state were considered statistically altered proteins and were further subject to gene ontology analysis.

The SRM data in this experiment were processed in Skyline (version 1.30) (26). Selection of chromatogram peaks for quantitation was manually verified. Peak smoothing method Savitzky–Golay was used. Chromatogram extracted peak areas for transition ions were summed up to yield total peak areas. The ratio of the total peak areas represented the abundance ratio of the peptides. Peptide ratios of each protein were further averaged to estimate the protein fold changes. The quantitation normalization was based upon equivalent total injection amount (1 μg) in each SRM run. A quantitation linear range of four orders of magnitude was shown (down to femtomole) (Fig. S1).

RESULTS

Evaluation of P. aeruginosa Proteome Quantitation Methods—Spectral Counting and Selected Reaction Monitoring (SRM)

We first evaluated the reliability of our proteome quantitation methods. Spectral counting quantitation allows broad coverage of P. aeruginosa proteins and was used to identify potential protein targets. More than 1,000 P. aeruginosa proteins were examined in these assays (Fig. 1A). The Exponentially Modified Protein Abundance Index (emPAI) (25) values of 921 proteins commonly observed in all cell samples were used to calculate the fold change for each protein. The linear quantitation range of emPAI for the P. aeruginosa proteome was assessed with bovine serum albumin (BSA) spike-in assays, and a linear range over three orders of magnitude (down to 1:10,000 dilution w/w) was observed for BSA proteins (Fig. S1A). The detected coefficient of variation (CV) of emPAI for these proteins appeared protein-dependent as one might expect and scored a median CV of ~40% (Fig. S2). The majority of protein emPAI ratios localized at the center of the scatter plot (log2 1 = 0), while the proteins with altered abundance appeared to be consistent between treatments, showing enrichment in Quadrant I and III of the Cartesian plane (Fig. 1B).

Selected reaction monitoring (SRM) achieves high specificity and accurate quantitation and was used for validation of targets identified by spectral counting. The linear quantitation range of SRM was also assessed by BSA spike-in assays, a linear quantitation range of four orders magnitude was achieved for BSA peptides (Fig. S1B), which is consistent with other reports (27, 28).

To determine if the protein-level changes measured by spectral counting or SRM were consistent, quantitation data of a set of 10 P. aeruginosa proteins (including 49 SRM peptide assays) at different tobramycin treatment conditions measured with both methods were compared. A general correlation (R2 = 0.61) was observed (Fig. S3). Therefore, emPAI spectral counting and SRM provide consistent measurements for P. aeruginosa proteins and allow us to systematically evaluate the proteome response of P. aeruginosa to tobramycin antibiotic treatment.

Concentration- and Time-Dependent Proteome Changes of P. aeruginosa in Response to Tobramycin Treatment—Since we are interested in the adaptive proteome response as a mechanism in protecting cells from antibiotic killing, P. aerugi-

To validate the tobramycin dosage effects on the observed proteome response, we used SRM to analyze the dosage-dependent levels of a number of these proteins (Fig. 3A–3C). The proteins can be categorized into three groups based on their biological functions, heat shock proteins, proteases, and amino acid catabolic enzymes (29). Heat shock proteins (IbpA, ClpB, DnaJ, DnaK, GrpE, and HtpG) were significantly
increased by tobramycin at all three dosage levels, with the
increases at the higher dosage levels (0.5 and 1.0 μg/ml) most marked. IbpA was again observed with largest fold change,
with 84-fold induction at 0.5 μg/ml and 95-fold at 1.0 μg/ml
tobramycin treatment, consistent with the quantitation from
spectral counting. For the proteases (PA0779, HslU, and
HslV), larger increases were also observed at the higher dos-
age treatment (0.5 and 1.0 μg/ml), while FtsH and PA1803
( Lon protease) exhibited similar increases at all three dosage
levels. For the amino acid catabolic enzymes, only DapB was
significantly increased at all three tobramycin concentrations,
while DhcA, DhcB, BkdB, and Sdr were significantly increased
only at 0.1 and 0.5 μg/ml treatments. For the control proteins
(TufA, Tig, and PolA), no significant abundance level change
was observed at any dosage treatment (Fig. 3D).

To interrogate the time dependence of inducible proteome changes, we used SRM to measure the tobramycin-induced proteins (IbpA, ClpB, DnaK, PA0779, and HslU) at the time points of 0, 15, 60, 120, and 360 min after 1.0 μg/ml treatment (Fig. 2C). Interestingly, for all five proteins, the rate of increase was most marked from 15 min to 60 min after tobramycin treatment and leveled off at 120 and 360 min. The temporal characteristics of IbpA increase resembles that seen after heat treatment (30). Again, the control proteins TufA and Tig did not significantly change.

We compared the proteome changes with published tran-
scriptome results (18) in order to further comprehend the
temporal regulation of proteome response. The transcriptome
analysis by Lee et al. (18) was performed with similar tobra-
mycin treatment conditions (1 μg/ml tobramycin 15 min treat-
ment, OD_{600} = 1.0 MPAO1 cells) as in the present study so provided a useful reference fold change of RNAs. As shown in Fig. S4, 11 genes were found to be tobramycin-induced in both transcriptome and proteome analyses, including seven of the genes that corresponded to proteins showing the greatest increase in proteome changes (Figs. 1C and 2A): PA0779, DhcA, BkdB, IbpA, ClpB, PA4759-PA4762, HslU-V were up-regulated, consistent with the results in the dosage treatment shown in Fig. 1C. (B) Inset of the proteome landscape showing time-dependent changes of IbpA and PA4759-PA4762. (C) Selected reaction monitoring (SRM) analysis for proteins that were dramatically increased in the tobramycin time course treatment. The SRM protein abundance ratio is shown, which represents the average of the peptide ratios from the protein. Interestingly, rapid increase of heat shock proteins and proteases occurred from 15 min to 60 min after tobramycin treatment, and the abundance level sustained till 360 min (last time point measured).

Additional increase suggests the importance of protein-level regulation of tobramycin response in \textit{P. aeruginosa}.

**Functional Enrichment of Tobramycin-Induced Proteins in \textit{P. aeruginosa}**—To further define the proteome response induced by tobramycin, we used a one-way unpaired \textit{t} test (also known as a one-way ANOVA test) to distinguish statistically significant changes of \textit{P. aeruginosa} protein levels in different treatment states. In addition to the statistical cutoff (\(P \leq 0.05\)), proteins with significantly altered levels were also required to be repetitively quantified minimally by three unique peptides from at least two of the seven replicates in dosage treatments or two of the five replicates in time course treatments. Proteins that were found by spectral counting with statistically significant level changes but lower than twofold changes were further validated by other quantitation methods (Fig. S5).

Using this approach, as many as 200 proteins appeared with statistically significant altered levels in different tobramy-
cin treatment states. Consistent with SRM quantitation that showed tobramycin dosage- and time-dependent effects on individual protein level changes (Figs. 2C and 3A, 3B, and 3C), the global proteome induction also showed systematic tobramycin dosage of all three dosages, and the extent of increase was more dramatic at the higher dosages 0.5 and 1.0 μg/ml. Amino acid catabolic enzymes DhCA, DhCB, BkdB, and Sdr were significantly increased at 0.1 and 0.5 μg/ml dosages, and enzyme DapB was significantly increased in all three dosages. Control proteins TufA, Tig, and PolA did not show significant fold changes in tobramycin treatment. Three to five SRM peptides (each containing 5–8 transition ion quantitation) were used to quantify each protein. The diamond represents the measured fold change for each peptide. The black line indicates the fold change of the protein by averaging the fold change of all the SRM peptides. (E)-(G) Scatter plot showing the induction for heat shock proteins, proteases, and amino acid catabolic enzymes. The spectral counting data log2 (fold change) of Tob0.1/CK (x axis) and Tob0.5/CK (y axis) are shown in (E) and (F), and log2 (fold change) of Tob0.1/CK (x axis) and Tob0.5/CK (y axis) are shown in (G). The whole quantified proteome is provided in blue diamond as background. The heat shock proteins are highlighted in red, proteases in orange, amino acid catabolic enzymes in purple. Proteins in these three pathways contributed most of the largest fold changes in the proteome.

Interestingly, examination of the top 30 proteins with greatest fold changes from the three tobramycin dosage treatments indicated that very few proteins were common among the three dosage levels, and the proteins that were common in two dosage levels were more likely to be shared between more similar doses (i.e. 0.1 and 0.5 μg/ml or 0.5 and 1.0 μg/ml) (Fig. 5A). These results suggest that bacterial responses become less similar as the differences in the tobramycin dose increase. Because significant proteome induction was observed at the very low dosage levels (0.1 μg/ml, 214 nM, 1/10 MIC), it also illustrates that P. aeruginosa cells can sense a very low amount of tobramycin molecules, supporting the notion that cells first exposed to lower-level tobramycin treatment could yield acclimation phenotypes.

We next characterized the gene ontology of proteins that were observed to increase with tobramycin (Fig. 6A-6C). At higher tobramycin dosages of 0.5 and 1.0 μg/ml, protein...
folding function (heat shock proteins) and proteolysis function (proteases) were significantly overrepresented as compared with other gene ontology categories. These functions were temporally up-regulated at 60, 120, and 360 min with 1.0 g/ml tobramycin treatments. The up-regulation of protein folding and proteolysis functions highlights the importance of protein quality control after higher tobramycin exposures (21, 31). At lower dosages of 0.1 and 0.5 g/ml, amino acid metabolic/catabolic (but not biosynthetic) pathways were statistically overrepresented. Thus, different functional activation was observed dependent on tobramycin treatment dosages, and one would anticipate differences in cell phenotypes when specific subsets of proteins were up-regulated (32).

To further interrogate the response of amino acid metabolic enzymes, the metabolic subpathways for different amino acid families were analyzed (Fig. 6B). Interestingly, overrepresentation for the amino acid metabolic pathway was largely due to the catabolic pathways rather than the synthesis pathways. In terms of the amino acid family, the induction was common to enzymes in all five families, but the increases in the aromatic and branched chain amino acid families were more pronounced than the other families and achieved level changes with $P \leq 0.01$. Such increases were most apparent with the low level of tobramycin treatment 0.1 $\mu$g/ml.

To characterize the functional relationship of tobramycin-induced proteins, we analyzed the gene coregulation referenced from the STRING database (33). The induced proteins observed with 0.5 $\mu$g/ml tobramycin dosage are shown in Fig. 5B. Fourteen proteins related to heat shock and proteases functions were represented, and they were tightly connected in the network. Amino acid metabolic enzymes PA1999–2001 (DhcAB, AtoB) in carnitine catabolism, PA2013–2015 (LiuABC) in leucine degradation, and PA2247–2250 (BkdA1A2B, LpdV) in valine degradation (29) were also significantly induced. Although many carbon metabolic enzymes were also increased by tobramycin, the enrichment of the pathway was not statistically significant (Fig. 5B), considering the large base number of carbon metabolic enzymes in *P. aeruginosa* genome.

To verify the widespread increase for heat shock proteins, proteases, and amino acid catabolic enzymes, we examined the protein fold change of all the proteins quantified in the three pathways. As shown in Figs. 3E-3G, proteins in the three pathways contributed most of the highest fold changes in *P. aeruginosa* proteome, indicating that the three pathways are critical to *P. aeruginosa* tobramycin response.

As a complementary analysis, we also analyzed the tobramycin repressed proteome in *P. aeruginosa*. Proteins with levels that decreased during tobramycin treatment also showed dosage and time dependent effects (Fig. 4). Those proteins were functionally enriched in protein synthesis, nucleotide metabolism, tricarboxylic acid (TCA) carbon metabolism and energy derivation, and electron transport activities (Fig. S6). Gene coregulation analysis demonstrated that the
down-regulated proteins were functionally connected (Fig. S7).

Tobramycin Sensitivity Phenotypes of ibpA Single and Double Mutants—Because IbpA levels were increased by the greatest amount in the observed proteome response to tobramycin, we constructed ibpA gene-inactivation mutants to determine if the absence of IbpA proteins may result in changes of P. aeruginosa tobramycin sensitivity. Surprisingly, neither ibpA-lux inactivation mutant nor ΔibpA in-frame deletion mutant exhibited significant changes in tobramycin sensitivity compared with MPAO1 WT (Table I and Fig. S8). This observation suggested that either IbpA is not directly involved in tobramycin resistance or the function of IbpA is compensated by other heat shock chaperones (34), leading to no obvious changes in tobramycin sensitivity. To examine these possibilities, we further constructed the double mutants of ibpA with clpB, (another heat shock protein) or PA0779 (lon protease) or hslV (ATP-dependent protease subunit), all of
which showed significantly increased protein levels with tobramycin treatments (Figs. 3A and 3B) and exposure time (Figs. 2A and 2C).

Detectable increases in tobramycin sensitivity were observed for most of the ibpA double mutants. The double mutants ibpA/hslV exhibited tobramycin MIC of 0.25 μg/ml, which was twofold more sensitive as compared with single mutant ibpA and MPA01 WT (Table I and Fig. S8). Two of the ibpA/hslV strains also showed twofold enhancement in tobramycin sensitivity compared with hslV single mutant. For double mutants ibpA/clpB, ibpA/PA0079, tobramycin MIC of 0.5 μg/ml was identified. Although the extent of tobramycin sensitivity change was relatively small, close examination of colony lawn density showed that ibpA/clpB and ibpA/PA0079 strains were almost absent at tobramycin MIC of 0.5 μg/ml, whereas a clear thin lawn was still observed for single mutants ibpA, clpB, and PA0079 and MPA01 WT at the same plates (Table I and Fig. S8). These sensitivity differences were reproducible for different transformants of double mutants tested. Thus, ibpA/clpB and ibpA/PA0079 double mutants were also more tobramycin sensitive compared with the single mutants. The MIC results support the hypothesis that Ibpa, as well as ClpB, HslV, and PA0779 proteins, indeed play a role in tobramycin resistance in P. aeruginosa cells; however, some of these chaperones and proteases may share overlapped function, which preclude the observation of strong tobramycin sensitivity changes for single-gene inactivation mutants.

**DISCUSSION**

In this study, we investigated the dynamic concentration- and time-dependent proteome response of P. aeruginosa to the aminoglycoside antibiotic tobramycin. Marked proteome changes were observed after 60 min of tobramycin exposure and in response to the dosage as low as 0.1 μg/ml (1/10th MIC). The concerted proteome changes led to the functional induction of heat shock proteins and proteases at higher dosages (1.0 and 0.5 μg/ml) and amino acid catabolic enzymes at lower dosages (0.1 and 0.5 μg/ml). We further showed that inactivation of proteome markers ibpA/clpB, ibpA/PA0779, or ibpA/hslV was observed with increased tobramycin sensitivity changes in P. aeruginosa, which supports the notion that proteome response indeed has effects on antibiotic resistance in P. aeruginosa.

Our proteomics approach complements previous genetic analyses from the P. aeruginosa transposon mutant library (16, 18, 35, 36) and reveals the dynamic changes of many...
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**TABLE I**

*Summary of tobramycin MIC assays for P. aeruginosa mutants*[^1]

| Genotype and alleles | Strains | Tob MIC (µg/ml) |
|----------------------|---------|----------------|
| WT                   | MPA01   | 0.5–1          |
| ftsH gene deletion   | ΔftsH   | 0.03–0.06      |
| ibpA in-frame deletion | ΔibpA | 0.5–1          |
| ibpA gene inactivation with lux insert | ibpA-lux | 0.5–1 |
| Control containing lux insert | PKH181 | 0.5–1 |
| Control for Tn insertion | PA3303 | 0.5–1 |
| clpB single mutant, Tn 1076(2565) | clpB (PW8651) | 1 |
| clpB single mutant, Tn 1076(2565) | clpB (PW8652) | 1 |
| PA0779 single mutant, Tn 2245(2400) | PA0779 (PW2411) | 1 |
| PA0779 single mutant, Tn 1351(2400) | PA0779 (PW2414) | 1 |
| PA0779 single mutant, Tn 1367(2400) | PA0779 (PW2413) | 1 |
| hslV single mutant, Tn 223(534) | hslV (PW9487) | 0.25 |
| hslV single mutant, Tn 168(534) | hslV (PW9485) | 0.5 |
| hslV single mutant, Tn 244(534) | hslV (PW9486) | 0.5–1 |
| double mutant ibpA/clpB | ibpA/PW8651 (1,2,3) | 0.5, 0.5, 0.5 |
| double mutant ibpA/clpB | ibpA/PW8652 (1,2,3) | 0.5, 0.5, 0.5 |
| double mutant ibpA/PA0779 | ibpA/PW2411 (1,2,3) | 0.5, 0.5, 0.5 |
| double mutant ibpA/PA0779 | ibpA/PW2413 (1,2,3) | 0.5–1, 0.5, 0.5 |
| double mutant ibpA/hsIV | ibpA/PW9487 (1,2,3) | 0.25, 0.25, 0.25 |
| double mutant ibpA/hsIV | ibpA/PW9485 (1,2,3) | 0.25, 0.25, 0.25 |
| double mutant ibpA/hsIV | ibpA/PW9486 (1,2,3) | 0.25, 0.25, 0.25 |

[^1]: The sequence-verified transposon insertion mutants (Tn) are as reported in Held et al. (17), and information of mutant-specific insertion sites (gene lengths), e.g. 1.076(2,565) and library names, e.g. clpB (PW8651) are indicated in the table. Two lines of ibpA/clpB mutants, three lines of ibpA/PA0779, and ibpA/hsIV were examined. For each double mutant line, three independent transformants (1,2,3) were assayed. Tobramycin MIC was defined as the lowest antibiotic concentration preventing the lawn growth of the spotted cells. A listing of a range of concentration levels indicates that a very thin lawn was observed at the lower concentration and this lawn was entirely abolished at the higher concentration in the range. See Figure S8 for additional details.

...essential candidate genes during tobramycin treatment. For example, protein levels for essential candidate genes DnaK, GrpE, GroEL, and GroES heat shock proteins were significantly increased at dosages 0.5 and 1.0 µg/ml and time points 60, 120, and 360 min. Likewise, essential metabolic enzymes Dhca, Dhcb, and DapB were significantly up-regulated by 0.1 and 0.5 µg/ml dosages. The observed protein-level changes support the hypothesis that many of these essential genes are not only indispensable for *P. aeruginosa* normal growth but also play important roles in tobramycin response.

Adaptive resistance of *P. aeruginosa* toaminoglycoside antibiotics has been described both *in vivo* and *in vitro* (13–15). It is associated with enhanced bacterial drug resistance and reduced clinical treatment efficacy (15). Some previously described mechanisms of adaptive response involved membrane impermeability (37) and the up-regulation of efflux pump MexXY (38). We hypothesize that such adaptive resistance may also involve other changes at the proteome level. Results presented in this manuscript demonstrate that the *P. aeruginosa* proteome response is dependent on the tobramycin levels experienced by cells, with induction of different groups of proteins being observed with exposure to various subinhibitory levels (0.1–1 µg/ml) of tobramycin. Thus, *P. aeruginosa* sense and respond differently to tobramycin, depending on the antibiotic concentration, supporting the adaptive resistance concept (12).

The observed time scale of proteome response is likely sufficient to produce functional impact in antibiotic defense. For example, time-lapse confocal microscopy analyses indicate that exopolysaccharide layers of biofilms can delay the biocide penetration to the central cell clusters up to 60 min (and in some cases, even longer) (10, 11, 39). In addition, CF patients that inhaled a second dose tobramycin 1 h after an initial dose also experienced the time-dependent decrease in drug efficacy (15). This effect was hypothesized to be resultant from induced adaptive resistance of *P. aeruginosa in vivo*. Thus, this time dependence of bacterial adaptive resistance is consistent with the observed time dependence in proteome changes in the present study. We also show that prolonged exposure of *P. aeruginosa* to tobramycin up to 6 h is associated with sustained proteome acclimation, during which time adaptive resistance also occurs (13–15).

Distinct proteome subsets were induced when *P. aeruginosa* cells were exposed to increasing concentrations of tobramycin. Such concentration-dependent changes underscore the diverse mechanisms in *P. aeruginosa* adaptive response. Widespread induction of heat shock proteins and proteases were observed with 0.5 and 1.0 µg/ml tobramycin treatments, consistent with previous transcriptome analysis.
P. aeruginosa Proteome Response to Tobramycin

(31, 40). Recent Escherichia coli proteome analysis of streptomycin treatments also highlights the roles of heat shock chaperones and proteases (41, 42), suggesting the response may be conserved among bacterial species and aminoglycoside drugs.

In particular, heat shock protein IbpA was the highest fold increased protein in the present study. Inactivation of ibpA did not yield significant tobramycin MIC changes. However, inactivation of two heat shock proteins/proteases ibpA/clpB, ibpA/PA0779, or ibpA/hslV led to increased tobramycin sensitivity changes in P. aeruginosa. The lack of strong changes in tobramycin sensitivity for IbpA may be owing to the over-lapped function of IbpA with other heat shock chaperones and proteases in P. aeruginosa. In E. coli, IbpA was found to perform concerted actions with ClpB and DnaK-DnaJ-GrpE to reverse protein aggregation induced by high temperatures (34, 43).

In addition to the induction of heat shock chaperones and proteases, changes in amino acid metabolic and biosynthesis functions were observed with tobramycin treatments at the lower dosages 0.1 and 0.5 µg/ml and longer time points 120 and 360 min. These include the up-regulation of essential candidate genes of dhcA and dhcB, which are dehydrocarnitine CoA transferase subunits involved in metabolism of the amino acid carnitine, and dapB, which is a 4-hydroxy-tetrahydrodipicolinate reductase involved in lysine biosynthesis. Consistent with our findings that show amino acid metabolic enzyme levels were altered during tobramycin exposure, the interaction effects of amino acids and aminoglycoside antibiotics have been reported. For instance, supplementing 0.4% L-arginine in tryptic soy agar plate enhances the tobramycin killing of P. aeruginosa biofilm colonies over 10-fold (44). Conversely, the presence of 20 mM cadaverine (a decarboxylation product of lysine amino acid) promotes P. aeruginosa resistance to aminoglycoside kanamycin and gentamycin by fourfold (45), while suppressing its resistance to carboxypenicillins (46). Although much still needs to be understood, the results demonstrated here show that bacterial cells exposed to low levels of tobramycin exhibit increased levels of enzymes that metabolize and synthesize amino acids that could alter drug sensitivity. If so, manipulation of amino acid homeostasis pathways may provide new ways to modulate proteome response in P. aeruginosa and improve antibiotic treatment effects.

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