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The Development of Ciprofloxacin Resistance in *Pseudomonas aeruginosa* Involves Multiple Response Stages and Multiple Proteins▽†‡

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Microbes have developed resistance to nearly every antibiotic, yet the steps leading to drug resistance remain unclear. Here we report a multistage process by which *Pseudomonas aeruginosa* acquires drug resistance following exposure to ciprofloxacin at levels ranging from 0.5× to 8× the initial MIC. In stage I, susceptible cells are killed *en masse* by the exposure. In stage II, a small, slow to nongrowing population survives antibiotic exposure that does not exhibit significantly increased resistance according to the MIC measure. In stage III, exhibited at 0.5× to 4× the MIC, a growing population emerges to reconstitute the population, and these cells display heritable increases in drug resistance of up to 50 times the original level. We studied the stage III cells by proteomic methods to uncover differences in the regulatory pathways that are involved in this phenotype, revealing upregulation of phosphorylation on two proteins, succinate-semialdehyde dehydrogenase (SSADH) and methylmalonate-semialdehyde dehydrogenase (MMSADH), and also revealing upregulation of a highly conserved protein of unknown function. Transposon disruption in the encoding genes for each of these targets substantially dampened the ability of cells to develop the stage III phenotype. Considering these results in combination with computational models of resistance and genomic sequencing results, we postulate that stage III heritable resistance develops from a combination of both genomic mutations and modulation of one or more preexisting cellular pathways.

In the ongoing war between bug and drug, *Pseudomonas aeruginosa* is a frequent victor because it rapidly develops new defenses to any drug that is generated (31, 41). This is of grave concern for prevention and spread of infectious disease and is a significant mystery to bacteriologists. While there are a number of known resistance mechanisms that develop in *P. aeruginosa*, the mystery stems from how these are rapidly generated and accumulate in a population to quickly form high-level resistance to an antimicrobial drug after exposure. Finding solutions to inhibit the rise of resistance in *P. aeruginosa* is important because the organism is responsible for chronic lung infection in individuals with cystic fibrosis (CF) (6) or chronic obstructive pulmonary disease (COPD) (13, 15, 28), and it also accounts for nearly 10% of hospital-acquired infections (47, 52).

There is a small set of drugs commonly used to treat *P. aeruginosa* infection, including ciprofloxacin, tobramycin, gentamicin, ceftazidime, and imipenem. While *P. aeruginosa* has developed various levels of resistance to each of these, its response to ciprofloxacin is of particular interest because the drug is initially very effective, but *P. aeruginosa* rapidly acquires high-level resistance, rendering the drug impotent. In clinical isolates, approximately 30% of strains now present high-level ciprofloxacin resistance (31).

While there have been many studies of factors involved in ciprofloxacin resistance in *P. aeruginosa* (e.g., references 17, 22, and 48), the series of steps and molecular mechanisms that initially lead from bacteria being antibiotic susceptible to being drug resistant still remain unclear. This presents an obstacle to efforts directed at curbing the emergence of new drug-resistant strains and at prolonging the useful life span of existing or newly developed antibiotic drugs. We set out to examine the initial steps leading to ciprofloxacin resistance in *P. aeruginosa*, in order to ultimately develop strategies that may overcome *P. aeruginosa*'s proclivity for rapidly developing new defenses.

The primary factors uncovered in ciprofloxacin resistance to date are mutations of the DNA gyrase or topoisomerase IV (1, 16, 32, 51) and mutations in the efflux pump regulatory genes *mexR* and *nfxB* (19). Additionally, it has been noted that gene expression patterns are substantially altered after ciprofloxacin exposure (8), although the specific impact upon resistance is not yet known.

While the mutations uncovered have been associated with high-level resistance in isolates derived after the fact, significant questions remain unanswered. (i) Are these the only factors involved in high-level ciprofloxacin resistance? (ii) Does resistance arise in a single step or by a multistep process?

We set out to answer these questions by first examining the population kinetics for two strains (PAO1 and PAK) during

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exposure to ciprofloxacin to observe population responses to the drug. Observing an apparent multistage process of resistance development, we sought to uncover the specific mechanisms underlying the process. One branch of that effort was pursued using high-throughput sequencing to uncover the set of mutations involved in this process (unpublished data). In a second branch, we delved further into the altered patterns of gene expression previously observed using microarray analysis, by examining the impact upon protein expression. The results of proteomic analysis were followed by an analysis of the effects of transposon insertion mutants on several key proteins we identified. These efforts uncovered several intriguing observations, hinting that there may be more than meets the eye when it comes to the initial development of ciprofloxacin resistance in *P. aeruginosa*.

MATERIALS AND METHODS

Materials and equipment. Luria agar base Miller (LA) medium (TEKnova Co.) and Luria broth base Miller (LB; Acros Organics) were from Fisher Scientific (Pittsburgh, PA). Ciprofloxacin, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, iodoacetamide, and dithiothreitol were from Sigma-Aldrich (St. Louis, MO). The IPGphor for isoelectric focusing, IPG DryStrips (18 cm; pH 3 to 11 nonlinear), 2-D Pharmalyte pH 3 to 11, IPG buffer, a Typhoon 9400 variable mode imager, and ImageQuant software for image analysis were from GE Healthcare (Piscataway, NJ). Pefabloc SC was obtained from Roche (Mannheim, Germany). A Protean II xi 2-D cell was obtained from Bio-Rad (Richmond, CA). The Pro-Q Diamond phosphoprotein-specific stain SYPRO Ruby protein gel stain and the Live/Dead *Bac*Light bacterial viability kit were obtained from Invitrogen-Molecular Probes (Carlsbad, CA). The Eclipse E600 epifluorescence microscope was a product of Nikon Inc. Instrument Group (Melville, NY). The microplate reader, a SpectraMax M2, was from Molecular Devices (Sunnyvale, CA). Mass spectrometry for protein identification was carried out with an ABI 4800 matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometer located in the UNC/Duke Michael Hooker Proteomics Core Facility (Applied Biosystems, Foster City, CA).

Bacteria strains, media, culture conditions, and MIC assays. *P. aeruginosa* PAO1 was obtained from the University of Washington Genome Center. The University of Washington transposon mutant library was the source of all *P. aeruginosa* mutants used in this study (18). *P. aeruginosa* PAK (ATCC 53308) was provided by Matthew C. Wolfgang (University of North Carolina at Chapel Hill). *P. aeruginosa* PAK is known to carry approximately 98% of the *P. aeruginosa* PAO1 genes (50). All liquid cultures were grown at 35°C with aeration in a 500-ml Erlenmeyer flask with 50 ml of LB medium and supplemented with ciprofloxacin where indicated; the culture on LA medium with ciprofloxacin was grown for 48 h at 35°C and then CFU were counted. MICs were determined by Etest (AB bioMerieux, AB Biodisk), using the prescribed protocol. For the 96-well microtiter plate experiments used to assay MIC heterogeneity before and after ciprofloxacin exposure, MICs were determined using the broth dilution method (10). For the ciprofloxacin exposure assays, 0.5 ml of overnight culture was diluted into 50 ml of fresh Luria broth (1:100), incubated for 4 h to reach early stationary growth phase (1×10^9 to 2×10^9 cells/ml), and then exposed to ciprofloxacin. In all cases, viable cell counts were determined from at least two independent experiments by serial dilution plate counting on Luria medium agar after a 48-h incubation at 35°C.

Procedures for assaying heterogeneity of cellular responses to ciprofloxacin. Wild-type PAK cells were exposed to 0.125 μg/ml ciprofloxacin (1× the starting MIC) in liquid LB for 6 h or 20 h. The unexposed wild-type strain and the drug-exposed strains were then individually plated on LA medium (without ciprofloxacin) and grown at 35°C overnight to produce colonies. We then randomly picked 96 colonies from each plate (nonexposed, 6 h, and 20 h) and inoculated them into 96-well microtiter plates for culture overnight in fresh liquid medium (LB). The overnight bacterial culture of each well was adjusted with sterile broth to give a turbidity equivalent of an optical density at 600 nm of ~0.1. Then, a volume of bacterial suspension equal to the volume of diluted antimicrobial solution was added to each well of ciprofloxacin to measure the MICs (10).

Procedures for cellular protein extraction and 2D gel electrophoresis. For two-dimensional (2D) gel electrophoresis, in two biologically independent experiments the whole-cell extracts from wild-type *P. aeruginosa* PAK and the ciprofloxacin-resistant strain PAK-F5 were analyzed using the O'Farrell gel technique (34). Before 2D gel electrophoresis, all protein samples were cleaned according to the Pro-Q Diamond stain manufacturer's protocol (39) to minimize nonspecific staining due to phospholipids and other cell constituents. Approximately 300 μg of each protein sample was applied to IPG DryStrips (pH 3 to 11 nonlinear) for the first-dimension isoelectric focusing separation. IPGphor voltage profiles were as follows: 30 V for 390 V · h, 1,000 V for 1,000 V · h, 2,000 V for 4,000 V · h, 4,000 V for 6,000 V · h, 6,000 V for 12,000V · h, and 8,000 V for 14,000 V · h, with a total focusing of 38 kV · h. Prior to the 2D electrophoresis, the IPG strips were equilibrated as described by Görg et al. (14). The second-dimension separation was carried out by large-format 10% SDS-polyacrylamide gel electrophoresis using a Protean II xi 2-D cell (Bio-Rad). Gels were fixed (50% methanol–10% acetic acid overnight) prior to staining and visualization. Additional procedures are described in the supplemental material.

Multiplexed protein and phosphoprotein fluorescence labeling and quantitation. Fluorescent staining of 2D SDS-polyacrylamide gels was first performed using Pro-Q Diamond phosphoprotein stain and subsequently SYPRO Ruby protein stain (Invitrogen-Molecular Probes, Carlsbad, CA) following the manufacturer's instructions and our previously established methods (45).

In-gel digestion, MALDI-TOF/TOF mass spectrometry, and database searches. As described in Results, spots of interest were chosen based on changes in the D (ProQ Diamond) and S (Sypo Ruby) values along with the D/S ratio. The spots were excised using a 2D-iD robotic spot picker (Leap Technologies, Carrboro, NC) into 96-well microtiter plates for automated proteolytic digestion using an Investigator ProGest robot (Genomic Solutions, Ann Arbor, MI). The resulting mass spectrometry (MS) data were used to identify the proteins excised by peptide mass fingerprinting with tandem MS (MS/MS) using Mascot (36) and our in-house-developed Genome Fingerprint Scanning (GFS) software (12), searching against the Mass Spectrometry Protein Sequence Database (MSDB; version 20060831) and the *P. aeruginosa* PAO1 genome, respectively. Typical search parameters included two missed cleavages and 50 ppm tolerance for peptide mass fingerprinting. Proteins were identified based on producing results above the significance cutoff for the database search ($P < 0.05$; Mascot MS and MS/MS score above 74). See the supplemental material for further details on in-gel digestion and MALDI-TOF/TOF analysis.

Ciprofloxacin resistance development assays. Three single-gene transposon insertion mutants of *P. aeruginosa* PAO1, each deficient in the expression of one gene encoding a protein determined to be differentially expressed or modified in our analyses (strains 8773, 18230, and 631) were obtained from The University of Washington transposon mutant library (18). We also obtained two gene insertion mutants for use as controls (strains 30848 and 32477). Wild-type *P. aeruginosa* PAO1 and each of the mutant strains were inoculated at 1:100 into fresh medium (50 ml LB in a 500-ml Erlenmeyer flask) and incubated at 35°C for 4 h from overnight cultures. For each mutant strain, the starting MIC was determined by Etest, which was used to establish a concentration for ciprofloxacin exposure equal to 4× the MIC. Ciprofloxacin was added at time zero to each culture (after 4 h of subculture incubation), and population dynamics (cell death and recovery) were monitored for 48 h through viable cell counts at times 0, 3, 6,12, 24, and 48 h. Viable cell count data were recorded at each time point in at least two independent experiments. Ciprofloxacin resistance levels for the wild type and mutants were measured by Etest (MIC assay) after 24 and 48 h of ciprofloxacin exposure.

Procedures for modeling heterogeneous responses by normal curve fitting. Mathematica version 6.0 was used to produce normal (Gaussian) curves with the free parameters μ (mean) and σ (standard deviation). Each normal curve was then used to map a theoretical 96-well experiment to histograms using the same set of thresholds at which the heterogeneity assay results were measured ($0.125 < \text{MIC} < 0.250$ μg/ml; $0.250 < \text{MIC} < 0.500$ μg/ml; $0.500 < \text{MIC} < 1.000$ μg/ml; 1.000 μg/ml $< \text{MIC}$). The free parameters were manually adjusted to find the best fit between the actual data (see Fig. 2A, below), and the synthetic plots of the normal curve data with thresholds were applied for each of the distributions at 0 h, 6 h, and 20 h.

Simulation of ciprofloxacin resistance development kinetics in *P. aeruginosa*. The Berkeley Madonna software (R. I. Macey and G. Oster, University of California, Berkeley, CA) was used to simulate growth/death curves during prolonged ciprofloxacin exposure. The model was provided by Bruce Levin and colleagues from their program Model of Persistence and Mutation to Resistance (http://www.eclf.net/programs). The model is based on a Hill function response for the population to ciprofloxacin (40). More details, code, and model parameters are provided in the supplemental material.

DNA sequencing. Whole-genome DNA sequencing was performed and will be the focus of a separate manuscript, although we discuss one result from that work

here. Briefly, whole-genomic DNA samples from wild-type PAO1 and an isolated ciprofloxacin-resistant strain, PAO1-6, were sequenced using a Roche genome sequencer FLX system (454) in the High-Throughput Sequencing Facility at the University of North Carolina. We used the *P. aeruginosa* PAO1 complete genome (accession number NC_002516.2; GI 110645304) as a reference to assemble the genome and used the genome sequencer Reference Mapper (Roche).

Additional information regarding our methods are available in the supplemental material.

RESULTS

Response of *P. aeruginosa* to 48-h ciprofloxacin exposure. Starting with wild-type PAK and PAO1 strains, we assayed the minimal inhibitory ciprofloxacin concentrations (MICs) by Etest to establish a baseline. The resulting MICs were within the expected ranges, with the PAK MIC at 0.25 μg/ml ciprofloxacin and PAO1 at 0.5 μg/ml ciprofloxacin, corroborating previous research (3).

We focused our subsequent kinetic population analyses on *P. aeruginosa* cultures initially grown to stationary phase, because these cells were observed in previous experiments and our own studies to exhibit the greatest ability to survive exposure (2, 24). We examined the population dynamics in response to ciprofloxacin exposure at concentrations ranging from 0.5 to 8× the MIC in both strains PAO1 and PAK.

In each case, after exposure to ciprofloxacin, a significant fraction of the initial population was killed by the drug, which we term stage I of the antibiotic response, the "susceptible cell death" phase. This stage is the standard and expected response to an antibiotic exposure and lasts 6 to 12 h, as illustrated in Fig. 1A and B. This first stage has been well studied, including a microarray analysis of changes in gene expression (7, 9, 20), changes in protein profiles (5), and a mathematical model of the killing kinetics (49).

Following stage I, in every one of our experiments there was a surviving subpopulation of cells that remained after the majority had succumbed to death by ciprofloxacin (Fig. 1A and B, blue arrows). These cells displayed several characteristics in common with previously characterized drug-tolerant or persistent phenotypes (4, 11, 23–25).

Previous researchers have noted that "drug-tolerant" cells display little heritability from one generation to the next, indicating that the phenotype is not based on genetic changes or mutations. We examined whether this observation applied to our surviving cells by extracting them at various time points after ciprofloxacin exposure, subculturing those without ciprofloxacin, then testing their MICs. Their resulting MICs were 0.75 μg/ml, slightly elevated from the baseline PAO1 wild-type level of 0.5 μg/ml but not elevated enough to be considered resistant. We termed this population as stage II of the antibiotic response due to its similarities to the previously classified drug-tolerant (persister) phenotypes.

While the cell populations exposed to the highest levels of ciprofloxacin remained static and relatively unchanging over 48 h, we noted that at lower exposure levels a cell population would regrow starting after about 24 h in PAO1 and 12 h in PAK (Fig. 1A and B). We tested the heritability of drug susceptibility for these populations by extraction and subculture without ciprofloxacin, followed by another Etest. The subcultures taken from these reconstituted populations displayed

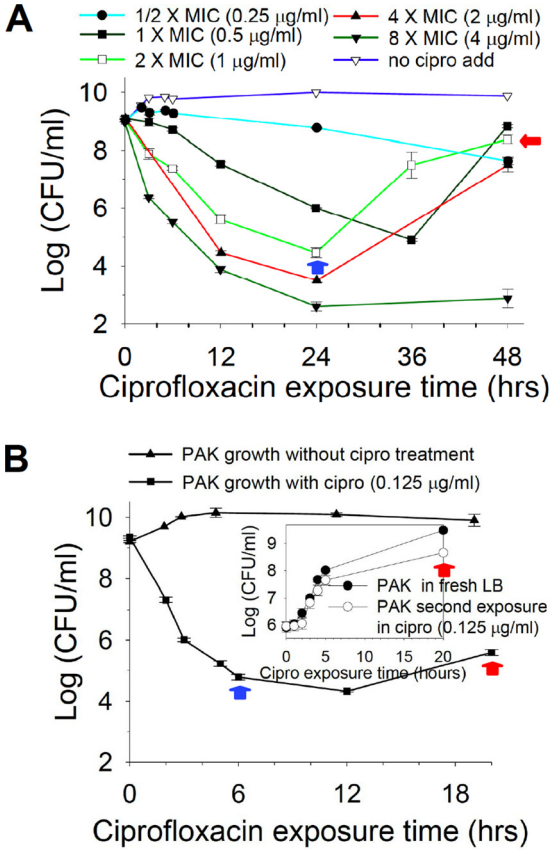

FIG. 1. *P. aeruginosa* growth kinetics during ciprofloxacin exposure. (A) Wild-type PAO1 cells derived from an early-stationary-phase culture were exposed to a range of ciprofloxacin concentrations from 0 to 8× the starting MIC of 0.5 μg/ml. (B) Wild-type PAK cells exposed to 0.5× the starting MIC of 0.25 μg/ml ciprofloxacin, compared to a nonexposed population. (Inset) Ciprofloxacin-exposed PAK cells subsequently regrown in fresh medium with ciprofloxacin for an additional 20 h (hollow circles), with growth curves similar to unexposed wild-type cells grown in fresh LB (dark circles). Blue arrows show time points at which MICs were reassessed without significant heritable increases, and red arrows show time points at which MICs were reassessed and showed significant increases in MIC values (to 8.0 μg/ml for PAO1 from the 2× starting MIC exposure and 2.0 μg/ml for PAK from the 0.5× starting MIC exposure). Data in are means ± standard errors from triplicate experiments.

substantially increased heritable MICs. For PAK, after 40 h of exposure at 0.5× the starting MIC (0.125 μg/ml), its population was reconstituted to $>10^8$ viable cells/ml and its heritable resistance increased by 8-fold to an MIC of 2 μg/ml (Fig. 1B, inset). For PAO1, after 48 h of ciprofloxacin exposure at 2× the starting MIC (0.5 μg/ml), its population reconstituted to $>10^8$ viable cells/ml, and its heritable resistance increased by 16-fold to an MIC of 8 μg/ml. Clearly these are a different type of cell than those we defined as stage II, based on their large increases in MICs and their rapid regrowth in the presence of ciprofloxacin. Hence, we labeled this as a third stage (stage III) in the development of heritable resistance. While previous studies have focused on the short-term antibiotic response in stage I and stage II (~2 to 6 h of exposure), our searches of the literature revealed very little information about the mecha-

nisms or processes underlying the rapid and remarkable development of resistance in these stage III cells (>12-h exposure).

Solid medium experiments to determine whether resistant cells were preexisting. The liquid culture experiments revealed a population of cells, termed stage III, in both PAO1 and PAK that regrew during ciprofloxacin exposure. To obtain evidence about whether these cells preexisted in the population before the first exposure to ciprofloxacin, we used solid medium experiments with large pregrown cultures of *P. aeruginosa*. We started with a 50-ml stationary-phase PAO1 culture of 10^9 cells/ml that was concentrated to 1 ml ($\sim 5 \times 10^{10}$ cells/ml), then plated these directly on a series of six LA plates, each impregnated with 1 μg/ml ciprofloxacin (2× the MIC). After 24 h of incubation on the ciprofloxacin-LA plates, we scraped the cells for resuspension in 3 ml of fresh LB and then grew them overnight. We then assayed the MICs by Etest, and they were in the range of 0.5 to 0.75 μg/ml, showing no evidence for the preexistence of a cell in the culture with substantially elevated resistance.

Distribution of ciprofloxacin resistance across distinct colonies. To study the distribution of ciprofloxacin resistance, we started with a 50-ml liquid culture of PAK grown to stationary phase and then collected 1-ml aliquots both before ciprofloxacin exposure and after 6 and 20 h of exposure (to 0.5× the starting MIC, 0.125 μg/ml). The washed and diluted cells were plated separately according to exposure time point and grown overnight on LA medium (without ciprofloxacin). We randomly isolated 96 distinct colonies from each of the distinct time point plates, transferring them individually into wells on a 96-well microtiter plate to measure their resultant pre- or post-exposure MIC. For each time point, the measured MIC distribution was heterogeneous (Fig. 2A) and could be approximately modeled with a normal distribution (Fig. 2B). As ciprofloxacin exposure time increased, the mean of the MIC distribution shifted higher, with the fitted normal distributions showing mean MICs rising from 0.29 μg/ml at 0 h to 0.63 μg/ml after 20 h, and their standard deviations increased from 0.08 μg/ml at 0 h to 0.21 μg/ml at 20 h. After 20 h of ciprofloxacin exposure, a few selected colonies displayed at least a 4-fold increase from the starting MIC, to >1 μg/ml, and many displayed more than a 2-fold increase. These data indicate that the mechanism(s) underlying stage III resistance is activated to various degrees throughout the population after ciprofloxacin exposure, not as a binary on/off resistance.

Mathematical modeling of tolerant and resistant populations of *P. aeruginosa*. We deployed a mathematical model of drug resistance provided by Bruce Levin and colleagues (http://www.eclf.net/) (40) to simulate the three-stage process proposed here (see the supplemental material for further details on the model). The model represents three populations of bacteria in an experiment: susceptible (S), tolerant/persistent (N), and resistant (R). We used the model to examine the question of whether resistant cells are preexisting in the population at the time of ciprofloxacin exposure or whether they develop resistance after exposure. In the initial model provided by Levin et al., the presence of 1 resistant cell/ml at time zero (ciprofloxacin exposure) was assumed, with the resistant cell(s) having a MIC of 20 μg/ml (40× the MIC of the susceptible PAO1 cells). Experiments were run assuming ciprofloxacin concentrations from 0 to 4 μg/ml and mutation rates as low as

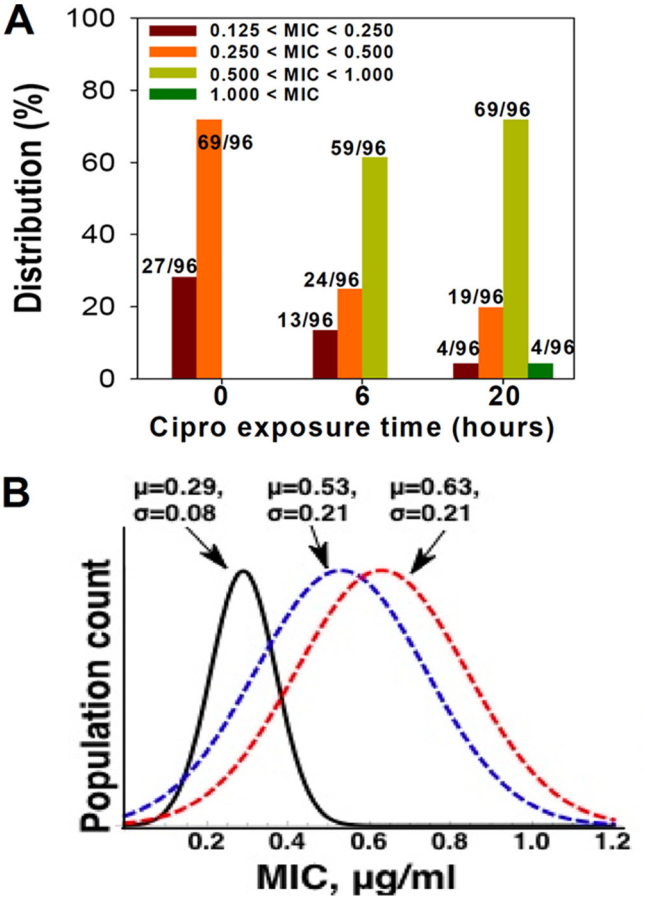

FIG. 2. Distribution of MICs for *P. aeruginosa* PAK for different ciprofloxacin exposure times. (A) Before ciprofloxacin exposure (0 h) and after exposure for 6 or 20 h, the measured MIC distributions at each time point are shown. (B) Normal curves fit to the heterogeneous ciprofloxacin responses shown in panel A. μ is the mean and σ is the standard deviation for the MICs of each curve. Data in panel A are means ± standard errors from triplicate experiments analyzing treatment with 0.5× the starting ciprofloxacin MIC (0.125 μg/ml).

10^{-18}. Regardless of mutation rate, for antibiotic concentrations (A) of ≤4 μg/ml, the population recovered to 10^8 cells/ml in under 27 h (Fig. 3A), with a high slope of recovery. Comparing the data for an A of 2 μg/ml from the model to the *in vitro* experiments at the same concentration, the model's population recovered much more quickly than the live cells.

In order to produce a model whose population recovered in the slower time frame shown *in vitro*, we had to remove the assumption that there were initially resistant cells in the population, by setting the initial resistant population *R* to $<10^{-6}$ cells/ml. We then adjusted the mutation rates for various simulations, and the models whose populations recovered on a time scale most similar to the lab experiment results had mutation rates in between 10^{-9} and 10^{-8}. These simulations agreed with our solid media experiments in indicating resistant cells are not likely present, nor are they necessary, to reproduce the *in vitro* population recovery results we observed.

Protein expression and regulation changes in stage III ciprofloxacin resistance. Previous studies have shown that, for very short ciprofloxacin exposure periods (<3 h), a wide array

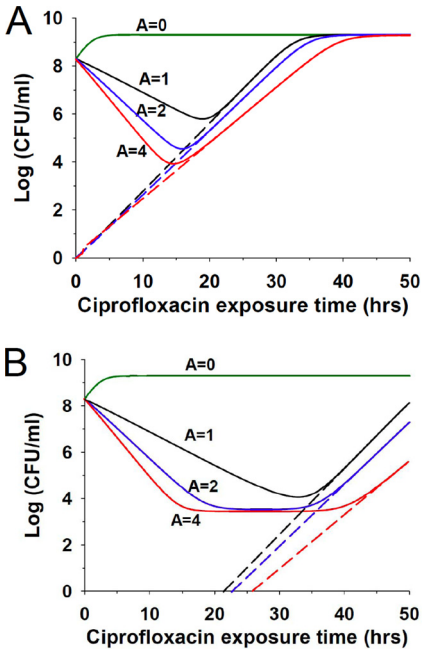

FIG. 3. Mathematical model of resistance development in *P. aeruginosa* based on computer modeling code developed for use in the Berkeley Madonna modeling environment, from Levin and colleagues (see the supplemental material for further information). We adapted the code to model the three-stage process postulated here. Three populations were assumed in this model: susceptible (S) cells, phenotypically tolerant (N) cells, and genetically resistant (R) cells, with densities expressed as bacteria per ml. (A) The initial model, starting with one resistant cell per ml (R = 1), one tolerant cell per ml, and the remainder as susceptible. Simulations were run for antibiotic concentrations (A) of 0 μg/ml up to 4 μg/ml. The mutation rate (mur) was 2×10^{-9}. (B) Simulations where the initial resistant cells were set to be effectively zero ($R = 10^{-6}$ cells/ml). Otherwise, we used the same parameters as in the previous simulations. Dashed lines show the resistant mutant population, and solid lines show the total population of live cells versus time.

of gene expression changes occur (7, 9, 20). To examine whether there are also substantial proteomic regulatory changes associated with longer exposure periods (>12 h), we compared protein expression from ciprofloxacin-susceptible wild-type PAK cells (MIC, 0.25 μg/ml) to a stage III derivative isolated after 20 h of ciprofloxacin exposure at 0.5× the starting MIC. These stage III cells had a new MIC of 2 μg/ml, an 8-fold increase over the wild type, and we hereafter refer to them as PAK-F5 cells. We analyzed extracted protein samples from two independent biological replicates by multiplexed 2D gel electrophoresis (43). We stained the proteins in the gels for phosphoprotein abundance (Pro-Q Diamond) and for total protein abundance (SYPRO Ruby).

Of the approximately 650 protein spots observed on the gels, three proteins displayed visually identifiable and repeatable differences in expression between PAK-F5 and unexposed wild-type cells. Two of those were differentially phosphorylated, having a 4-fold increase in the ratio of phosphorylated to total protein (Fig. 4) for PAK-F5 cells versus wild type. These proteins were identified by mass spectrometry as PA0265 (succinate-semialdehyde dehydrogenase [SSADH], encoded by *gabD*) and PA3570 (methylmalonate-semialdehyde dehydrogenase [MMSADH], encoded by *mmsA*). Surprisingly, neither of these had been previously identified as phosphoproteins in *P. aeruginosa*. They are putatively involved in amino acid metabolism and/or energy production (46). A third protein displayed 6-fold-higher total protein abundance in PAK-F5. It was identified by mass spectrometry as PA3539, a conserved hypothetical protein of unknown function, encoded by the gene *yaaA* (see Table S1 in the supplemental material).

Examining the role of the identified proteins in the survival of *P. aeruginosa* during ciprofloxacin exposure. We then asked the question: if we knocked out the genes encoding each of these proteins, would this affect survival, recovery, and/or resistance development of *P. aeruginosa* after ciprofloxacin exposure?

We obtained PAO1 mutants with transposon disruption of each of the encoding genes, along with two other mutant strains as controls, one without a functional *phoU* gene and the other without a functional Glu-tRNA (Gln) amidotransferase subunit A protein (encoded by *gatA*) (see Table S2 in the supplemental material). The *phoU* control mutant was chosen because a homologous protein was previously implicated in the formation of the persister phenotype (stage II) in *Escherichia coli* (27). The *gatA* control was chosen because its protein was visible on the same proteomic gels as the identified proteins yet appeared unchanged in expression between wild-type and resistant strains.

We determined the starting MICs for each mutant and then assessed their population kinetics after ciprofloxacin exposure at 2×, 4×, and 8× their starting MICs. The mutant strains displayed stage II surviving population ratios similar to the wild type and mutant controls (Fig. 5A, bottom panel; see also Fig. S1 in the supplemental material). However, the ability to reconstitute a stage III population in the continuing presence of ciprofloxacin was substantially muted by gene disruption (Fig. 5A, top panel; see also Fig. S1). The differences between strains were most apparent at 4× the starting MICs. The control mutants did not have similarly negative effects on stage III population recovery.

We then studied whether the ability to develop heritable resistance in the mutants (stage III) was inhibited. Each of the wild-type PAO1, control, and candidate strains was exposed to 4× and 8× the starting MICs of ciprofloxacin in liquid culture for 24 and 48 h. In wild-type PAO1, resistance increased 50-fold to a clinically significant level following 48 h of exposure at 4× the starting MIC (Fig. 5B). In the *gatA* control mutant, the result was very similar. The *phoU* control mutant showed reduced resistance compared to the wild-type after ciprofloxacin exposure at 8× the MIC, but with exposure to 4× the MIC resistance developed to about 35× its starting MIC.

In contrast to both controls, each of the stage III-associated mutant strains showed dramatic decreases in the development of heritable resistance after exposure at either 4× or 8× the starting MIC. None of them achieved a clinically significant drug resistance level. The resistance inhibition was strongest with the *mmsA* mutant.

We measured whether the reduced abilities of the candidates to survive exposure was due to a pleiotropic growth defect. The candidate mutants did not present any significant growth defects prior to ciprofloxacin exposure (see Fig. S2 in the supplemental material). Growth defects were only apparent after ciprofloxacin exposure, upon stage III recovery.

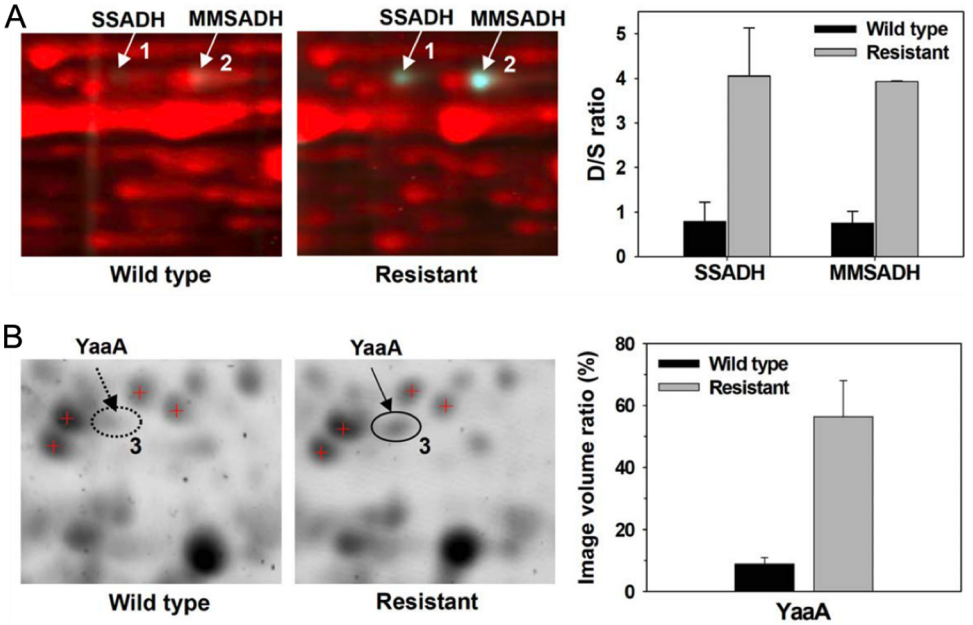

FIG. 4. Multiplexed 2D gel electrophoresis comparison of protein expression for wild-type PAK cells at early stationary phase versus the derivative PAK-F5 strain with ciprofloxacin resistance (at 8× the starting MIC, 2.0 μg/ml). (A) Proteins were visualized using the Pro-Q Diamond phosphoprotein stain (light blue) and SYPRO Ruby total protein stain (red). To the right, the ratios of the Pro-Q Diamond dye signal to the SYPRO Ruby dye signal (abbreviated as the D/S ratio) for two spots of interest are shown, as an approximate measure of the phosphorylation level relative to the total protein of that species. (B) Comparison of total protein concentration using SYPRO Ruby stain. The image volume on the right panel is the mean ± standard error of the ratio between the two gels for our identified protein, YaaA, representing a 6-fold expression level change compared to four nearby spots (labeled by the red cross) that were chosen as internal controls. The experiments in panels A and B were run in biological duplicates.

Effects of culture size on stage III development. In wild-type *P. aeruginosa*, we observed that the development of stage III resistance and the resulting population recovery was affected by culture volume (i.e., population size) and ciprofloxacin concentration (see Fig. S3A and B in the supplemental material). Examining this in detail, we measured PAO1 ciprofloxacin kill/regrowth curves in liquid culture volumes of 5, 25, and 50 ml, at both 2× and 4× the starting MIC.

At 2× the starting MIC, there was a clear recovery of the

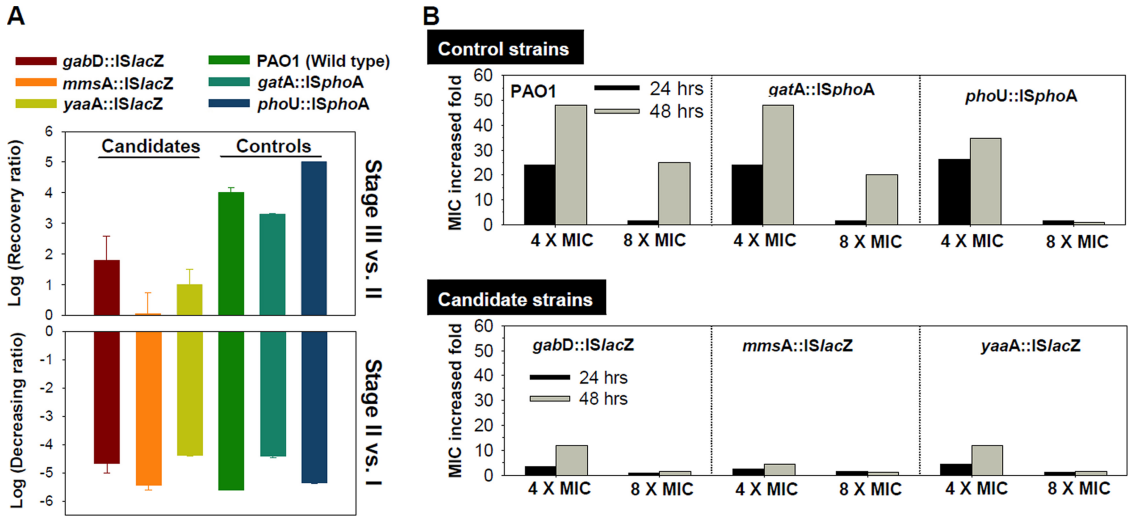

FIG. 5. Ciprofloxacin response profiles for candidate mutants versus controls. (A) PAO1 strain cultures starting with ~10^9 viable cells were then exposed to ciprofloxacin at 4× the starting MIC, as measured by Etest for each strain. (Bottom) Relative population reduction (stage II population versus stage I population) after 24-h ciprofloxacin exposure. Candidates and controls show similar population reductions at 24 h. (Top) Relative population recovery (stage III population versus stage II population) after 24 additional hours for the same cultures, based on the ratio of viable cell counts at 48 h versus 24 h. Data are means ± standard errors from triplicate experiments. (B) After 24-hour and 48-hour ciprofloxacin exposure in liquid medium, at 4× and 8× the starting MIC, control and candidate strains were retested to determine MIC levels relative to the starting level. The candidate strains responded with weak MIC increases relative to the control strains, and the *phoU* mutant also responded weakly after exposure to 8× its starting MIC.

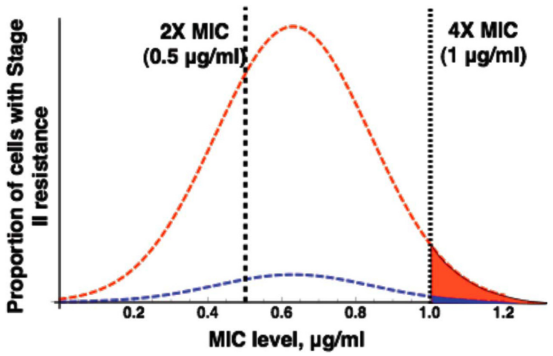

FIG. 6. Thought experiment for the effects of population size and ciprofloxacin concentration on the development of stage III resistance. The model shows sample normal curves based on the parameters derived for the 20-h exposure curve shown in Fig. 2B (μ, 0.63 μg/ml; σ, 0.21 μg/ml). The blue curve is scaled at 1/10 of the area of the red curve, illustrating the relative population sizes between a 5-ml culture and 50-ml culture. When cells were exposed to 2× the starting MIC (0.5 μg/ml), the majority of both populations developed resistance above this threshold, producing sufficient numbers of survivors to subsequently reconstitute the population (black hashed line on the left). However, when exposed to 4× the starting MIC, sufficient culture volume was required to allow reconstitution, as modeled by the black dotted line on the right. In this model, only the rare outlier members on the far right of the distribution will survive (red and blue shaded areas). When the volume is insufficient, there will not be enough cells above this threshold to survive and reconstitute the population (blue shaded area).

population with stage III resistance, regardless of culture volume (see Fig. S3A in the supplemental material). However, at 4× the starting MIC, only the 50-ml culture was reconstituted (see Fig. S3B). This result can be explained by our model of a normal distribution for MICs in the population (Fig. 2).

Depending on both the exposure level and population size, there may or may not be enough cells that develop stage II resistance above the survival threshold to reconstitute the population (Fig. 6). From the experiment shown in Fig. S3A and B in the supplemental material, we estimate the threshold to be about $\sim 10^5$ total cells that must survive in stage II in order to lead to a stage III population that reconstitutes.

Effects of other antibiotics. To investigate the response of both the wild-type PAO1 and the transposon mutants to other antibiotics, we assessed their MICs by Etest for a panel of antibiotics representing the fluoroquinolones, β-lactams, and aminoglycosides (see Table S3 in the supplemental material). For the quinolones, the mutant candidate strains exhibited 4-fold-reduced ciprofloxacin MICs and 8-fold-reduced levofloxacin MICs. For the β-lactam imipenem there was a 2- to 4-fold reduction in the MIC. There appeared to be little effect of the gene disruption mutants on the MICs for other classes of antibiotics.

DISCUSSION

Our results show strong evidence for a multistage drug adaptation process in *P. aeruginosa*. In stage I, the susceptible population (S) is killed off. This leaves stage II cells, surviving at a ratio of 1 in 10^4 to 1 in 10^5, and these cells appear to exhibit drug-tolerant phenotypes (N) that are non- or slow growing and do not display significantly increased MIC levels. Finally, after continuous exposure at moderate ciprofloxacin levels (<8× the MIC), a stage III population arises that is rapidly growing and exhibits substantially increased MIC levels. While a process of mutation appears to underlie the stage III cells (unpublished data that will be reported separately), it appears that nongenetic regulatory mechanisms may also play an important role in the resistance phenotype. In the PAK-F5 strain we identified three significantly altered proteins, two of which appear to have an effect via phosphorylation and appear to be essential for the progression to stage III.

We naturally wondered how these results could be related to previous studies that have shown a *gyrA* mutation as one primary molecular mechanism for ciprofloxacin resistance in *P. aeruginosa* (16, 32). Our whole-genome sequencing of PAO1 stage III cells confirmed that a *gyrA* mutation was present in the stage III cells. However, we found a variety of other mutations, none of which has been previously associated with ciprofloxacin resistance. We are presently assaying these mutations for their functions in ciprofloxacin resistance. Yonezawa et al. suggested that the *gyrA* mutation, while necessary for ciprofloxacin resistance, may not be sufficient for resistance. There is evidence that changes to other genes, such as *parC*, and the efflux pump regulator are necessary to produce high-level ciprofloxacin resistance in *P. aeruginosa* (16, 32, 51).

The multistage process of ciprofloxacin resistance in *P. aeruginosa* that we illustrated here raises a significant question: does the stage III resistance exist in the population before the bacteria are exposed to ciprofloxacin? One reason for thinking this is that the population sizes are vastly larger before exposure to ciprofloxacin than after. It might be argued that it would be improbable for mutations to arise out of the relatively small stage II population to produce stage III.

Yet the evidence all points to a different scenario: that the mutation(s) and regulatory changes evident in stage III arise directly from the stage II population, not from a preexisting mutant in the population. The evidence also indicates that the process is stepwise and heterogeneous, not a single, large jump to a higher resistance level. While this result is surprising when considered in the context of current theories, there is support for higher mutation rates in ciprofloxacin-exposed cells via the SOS response (29). When mutation rates are increased artificially via a *mutS* deletion, PAO1 can develop high-level ciprofloxacin resistance on the order of 1 in 10^6 cells per generation (35).

This is compatible with our results that demonstrated a required volume of cells of over 20 ml to develop stage III resistance. The stage II population cells are present at 10^4 to 10^5 cells per ml, and therefore, when the volume is 20 ml, we had a total cell number of $\approx 10^6$ in the culture. If we speculate that mutation of a tolerant cell to a resistant cell is occurring at a rate (mur) of 10^{-7} per hour (with ~2 generations per hour), we would expect the following: total cells × mur × 10^6 cells × 10^{-7} mutations/h × $10^{1.5}$ h, which results in three resistant mutants obtained in the culture.

Since only one resistance mutant is initially required to produce a growing population of stage II resistant cells, this experiment would quickly produce a recovered population of resistant cells as soon as one of these resistant cells occurred.

However, if we consider the case of a 1-ml volume, in which

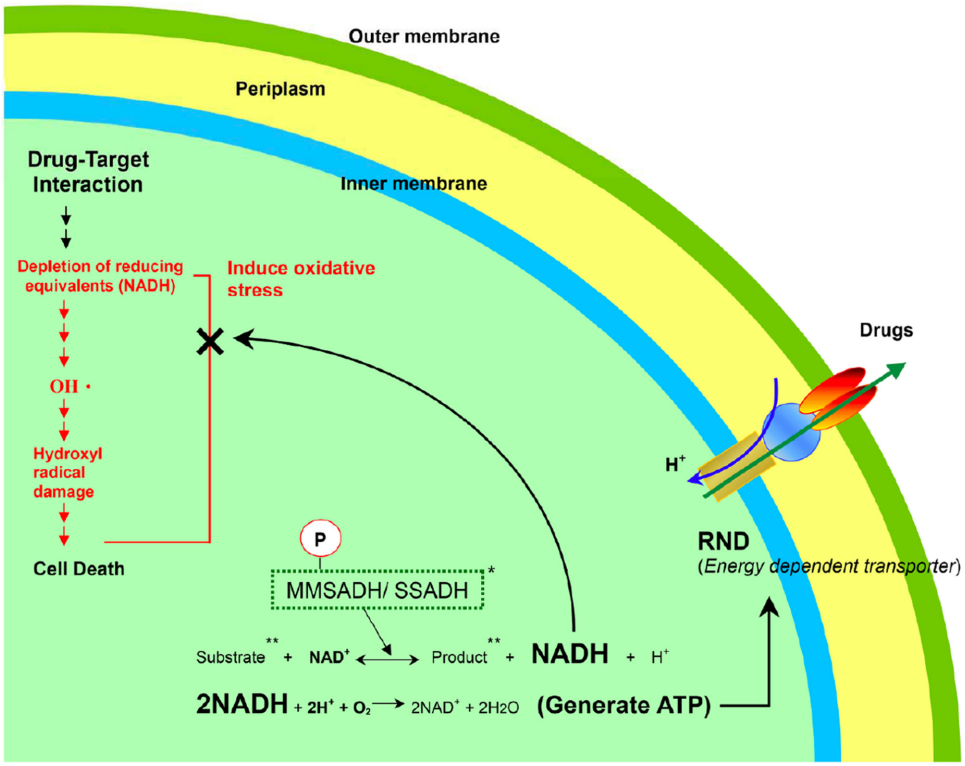

FIG. 7. Hypothetical model for the role of the phosphorylated proteins MMSADH and SSADH, characterized as playing a role in stage III resistance. Efflux pump systems are a known mechanism for innate drug resistance in *P. aeruginosa*, and this model shows the resistance-nodulation-division (RND) tripartite efflux system. RND transporters require energy for activation and operation. The identified proteins are both NAD-dependent aldehyde dehydrogenases that may be involved in electron transfer, supporting an energy conversion pathway to produce ATP. NADH is a by-product of the reactions catalyzed by both proteins, which may ameliorate oxidative stress induced by antibiotics like ciprofloxacin. The substrate for MMSADH is methylmalonate-semialdehyde plus coenzyme A (CoA), and the product is propionyl-CoA plus NADH. The substrate for SSADH is succinate semialdehyde, and the product is succinate plus NADH.

we did not observe resistance developing, then we get have the following: total cells $\times$ mur $\times$ 10^6 cells $\times$ 10^{-7} mutations/h $\times$ $10^{1.5}$ h, resulting in $\approx$0.3 resistant mutants. In other words, the volume and/or time span studied was not sufficient to produce the single mutant needed for growth and recovery of a stage III population.

The question remains whether this is an all-at-once mutation or whether multiple mutations must occur for resistance. Our evidence to date points to the production of stage III mutants being a multistep process, in which case the actual intracellular mutation rate may be higher, resulting in time for enough mutations to accumulate to produce a resistant mutant.

The notion that our nongrowing stage II cells produce mutants is supported by evidence that nondividing *E. coli* cells exposed to ciprofloxacin over 7 days produced adaptive mutations (42).

This leaves an open question: what role do the protein regulatory changes we observed play in stage III resistance? Our gene disruption results show that they play a vital role in resistance development for stage III, yet they are not necessary to produce stage II.

One possible explanation for their role would be in support of drug efflux pump activation, which is important for *P. aeruginosa* in ciprofloxacin resistance (30, 37, 44). The proteins MMSADH and SSADH may catalyze energy production, producing ATP that supports "energy-hungry" processes, like the efflux pumps (Fig. 7). These pumps, such as MexAB-OprM, operate using energy from hydrolysis of ATP or from the proton motive force derived from charge gradient across the cell membrane (33). If the pumps become activated in drug-resistant phenotypes, the energy production pathways feeding them would also require activation. There is evidence from the literature that an increased ATP/ADP ratio leads to increased ciprofloxacin susceptibility in wild-type *E. coli* cells (26), which contraindicates our hypothesis that these proteins produce more energy to support energy-hungry processes like efflux pumps and/or the SOS response. However, we're not sure that relating the effects of ATP/ADP ratios in wild-type *E. coli* to ciprofloxacin-resistant *P. aeruginosa* is a valid comparison.

An alternative hypothesis for the role of these proteins stems from the observation that two of them produce NADH as a reaction product (Fig. 7). NADH is produced in response to environmental stress (38) as a reducing agent that prevents oxidative damage. There is some evidence that major classes of bactericidal antibiotics, including the β-lactams, aminoglycosides, and quinolones, stimulate the production of hydroxyl radicals that may contribute to oxidation-mediated cell death (21). Therefore, we might postulate that NADH is upregulated by the phosphorylation of our identified proteins to buffer oxidative stress induced by ciprofloxacin.

Interestingly, these hypotheses are not mutually exclusive: both are compatible with all the data we have available. Could it be that evolution has wrapped two important responses to antibiotic resistance into one pathway? While the evidence is not yet strong, it is an intriguing possibility.

Regardless of the mechanism(s), the results make it clear that the development of resistance in *P. aeruginosa* is a multifaceted process involving three distinct stages: stage I, susceptible cell death; stage II, tolerant cell survival without growth or resistance; stage III, resistant cell development and population regrowth. In addition, the three proteins we found associated with heritable drug resistance demonstrate that there are preexisting cellular pathways in *P. aeruginosa* that act in support of genetic mutations, together producing heritable resistance to ciprofloxacin. This knowledge may ultimately lead to new strategies that prevent development of drug resistance to ciprofloxacin and other antibiotics.

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