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Evolutionary remodeling of global regulatory networks during long-term bacterial adaptation to human hosts

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The genetic basis of bacterial adaptation to a natural environment has been investigated in a highly successful Pseudomonas aeruginosa lineage (DK2) that evolved within the airways of patients with cystic fibrosis (CF) for more than 35 y. During evolution in the CF airways, the DK2 lineage underwent substantial phenotypic changes, which correlated with temporal fixation of specific mutations in the genes mucA (frame-shift), algT (substitution), rpoN (substitution), lasR (deletion), and rpoD (in-frame deletion), all encoding regulators of large gene networks. To clarify the consequences of these genetic changes, we moved the specific mutations, alone and in combination, to the genome of the reference strain PAO1. The phenotypes of the engineered PAO1 derivatives showed striking similarities with phenotypes observed among the DK2 isolates. The phenotypes observed in the DK2 isolates and PAO1 mutants were the result of individual, additive and epistatic effects of the regulatory mutations. The mutations fixed in the σ factor encoding genes algT, rpoN, and rpoD caused minor changes in σ factor activity, resulting in remodeling of the regulatory networks to generate generation of unexpected phenotypes. Our results suggest that adaptation to a highly selective environment, such as the CF airways, is a highly dynamic and complex process, which involves continuous optimization of existing regulatory networks to match the fluctuations in the environment.

chronic infection | epistasis | evolution of regulatory networks | microbial evolution | gene expression

Understanding how evolutionary forces shape organisms is of fundamental importance in biology. Experimental evolution studies—in which microbial populations evolve under defined conditions in the laboratory—have highlighted the importance of changes in regulatory networks in evolution of adaptive traits, and have frequently documented that regulatory mechanisms such as global regulators of gene expression are targets for adaptive evolution (1, 2). Similarly, other studies have linked variations in gene expression and in regulatory network structure to fundamental phenotypic differences among microbial species (3–5). Although these studies have provided novel insights into regulatory evolution and have shown that global regulatory networks are evolvable structures, the extent to which these observations relate to natural microbial populations remains less clear because few natural systems are amenable to direct observations of ongoing regulatory evolution on extended time scales.

Microbial infections in cystic fibrosis (CF) airways offer optimal opportunities to study microbial evolution (6), diversity (7), and interactions (8) in a natural setting, partly because of the systematic routine sampling from the ecosystem. Persistent airway infections with Pseudomonas aeruginosa are the major cause of morbidity and mortality for patients with CF. Long-term infection of the airways of patients with CF is associated with extensive genetic adaptation of P. aeruginosa, and, in an effort to understand the bacterial adaptive mechanisms that operate during persistent CF lung colonization, we recently characterized the genetic and phenotypic evolution of a persistent and transmissible P. aeruginosa clone (DK2) by genome sequencing and phenotypic profiling (6). This particular clone entered the CF Clinic in Copenhagen more than 40 y ago, adapted in one or a few patients, and subsequently spread to more than 40 other patients. This has made it possible to directly observe the evolutionary dynamics of the lineage on a time scale that covers as many as 200,000 bacterial generations of growth in the CF airways of a large number of infected patients.

Here we investigate the phenotypic consequences of five specific mutations sequentially fixed in global regulatory genes in isolates of the DK2 lineage during different stages of its long-term persistence in CF airways. We show that the regulatory mutations and their epistatic interactions contribute significantly to the major phenotypic changes observed in the DK2 lineage. The evolutionary history of the lineage reflects the transition from an opportunistic pathogen to a primary host-specific pathogen for patients with CF, and our results emphasize the role of continuing evolutionary modifications of regulatory networks in this process.

Results

We have recently determined the temporal sequence of genetic and phenotypic changes in the P. aeruginosa DK2 lineage that resulted in its successful establishment in the CF airways of a large number of patients from 1973 to 2008 (Fig. L4) (6). Remarkable phenotypic changes (measured as gene expression profiles and catalytic activity) were observed for DK2 isolates sampled during the early phase of entry into the CF environment (from 1973 to 1979), after which only minor phenotypic changes could be observed (Fig. 1B). The initial and rapid changes in the phenotype profiles suggest significant reorganization of gene regulatory networks and correlate with the sequential fixation of specific nonsynonymous mutations in the genes mucA, algT, rpoN, and lasR during the years until 1979.

The genes mucA, algT, rpoN, and lasR encode the regulators of three large and distinct global regulatory networks in P. aeruginosa: the Muca/AlgT (σE) network, the RpoN (σ54) network, and the LasR network. The mucA gene, which encodes the anti-σ factor Muca, is inactivated in DK2 by a frame-shift mutation (G430Δ, also known as mucA22), which results in full activation of the σ factor AlgT. The DK2-specific mutations in rpoN and algT are point mutations causing single amino acid substitutions in or adjacent to predicted functional domains of RpoN and AlgT, respectively (Fig. S1), whereas the lasR mutation is a whole-gene deletion resulting in complete loss of LasR.

Nature of Evolved Expression Changes in DK2 Lineage. To investigate the contribution of the four regulatory mutations to the evolved phenotype in contemporary DK2 isolates, we used allelic replacement to iteratively reconstruct the sequence of evolved DK2-specific


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alleles in reference strain *P. aeruginosa* PAO1. We initially observed that stepwise introduction of the evolved alleles resulted in colony morphology differences among the engineered strains (Fig. 2A), and that the morphological differences between WT and the quadruple mutant (Q mutant) in PAO1 paralleled the morphological transition observed in DK2 strains in which the four mutations were fixed (e.g., CF30-1979) relative to strains without the mutations (e.g., CF114-1973; Fig. 2B). To more precisely determine the contribution of the four mutations to the evolved phenotype of the DK2 isolates, we measured similarities in differential gene expression relative to PAO1 between the Q mutant and the DK2 isolate CF30-1979 by using the criteria specified in Materials and Methods. Strikingly, the comparison showed a large overlap of 700 genes, of which 631 were expressed in parallel by the two strains (Fig. 2C). With correction for the proportion of false positives appearing by chance in the expression profiles, the overlap of significant gene expression changes accounts for approximately 40% of the total transcriptome changes in CF30-1979. Given the inherent genomic differences between the DK2 strain and PAO1 (~18,000 SNPs) and the fixation of other mutations in the DK2 strain (6), the overlap was remarkable, and the finding clearly demonstrates that the four evolved regulator alleles contribute significantly to the evolved expression pattern observed in contemporary (i.e., post-1979) DK2 isolates. We notice, however, that the mutations in the PAO1 Q mutant did not always produce the same changes in magnitude of gene expression as observed in the clinical isolate, indicating that other mutations in the DK2 background may enhance the effect of the regulatory mutations.

**Expression Patterns and Interactions of Evolved Regulatory Networks.** To study in detail the regulatory effect of the evolved alleles, alone and in combination, we constructed three network-specific (NS) mutants: a mucAΔDK2 algTΔDK2 mutant, an rpoNΔDK2 mutant, and a lasRΔDK2 mutant. Global expression profiles were obtained for each mutant by measuring significant changes in gene expression relative to PAO1 (Dataset S1). As expected from the transcriptome analysis of the Q mutant, the expression profiles of each NS mutant involved differential expression (in both directions) of several hundred genes. Most changes were observed in the rpoN background (1,110 genes in total, with the highest gene enrichment observed in the functional classes *motility and attachment* and *chemotaxis;* Fisher exact test, *P* < 0.05), followed by the lasRΔDK2 background (637 genes in total, with the highest gene enrichment observed in the functional classes *protein secretion* and *secreted factors*) and mucAΔDK2 algTΔDK2 background (437 genes in total, with the highest gene enrichment in the functional class *amino acid biosynthesis and metabolism*). In total, we observed 2,184 expression changes in the NS mutants involving 1,745 unique genes, which point to a substantial regulatory overlap (439 genes) between two or more of the evolved networks.

To further explore the interaction between the evolved regulatory networks and to examine their combined effects on the transcriptome, we compared the expression profiles of the NS mutants to that of the Q mutant. As already indicated, the comparisons showed substantial overlaps between the evolved networks (Fig. 3A). Furthermore, clustering of the gene expression profiles revealed that most of the regulatory changes observed in the NS mutants were directly observable in the Q mutant background in terms of direct and additive effect of one or more
mutation(s) (Fig. 3B, cluster I). However, we also observed epistatic effects, as several genes in the Q mutant did not show the expression pattern otherwise expected from the patterns of expression in the NS mutants. For example, several genes (after false-positive correction) were up-regulated in one or more NS mutants (e.g., the mucA<sub>DK2</sub>algT<sub>DK2</sub> and lasR<sub>DK2</sub> mutants) without being expressed in the Q mutant (Fig. 3B, cluster II). The opposite scenario, in which genes were significantly expressed in the Q mutant but not in the NS mutants, was also observed (Fig. 3B, cluster III). These findings show that additive effects as well as epistatic interactions between the evolved regulatory networks contribute to the evolved expression pattern observed in the DK2 lineage.

**Modification of σ Factor Activity by Point Mutations.** We next investigated the regulatory effect associated with the single amino acid substitutions observed in the σ factors AlgT<sub>DK2</sub> and RpoN<sub>DK2</sub>, and measured how the modified proteins affect their respective regulons. We define the RpoN regulon as the number of genes, whose expression (directly and indirectly) depends on rpoN, and the AlgT regulon as the number of genes, whose expression (directly and indirectly) depends on the derepressed action of AlgT. The native regulons were determined by the gene expression profile of an rpoN knockout (KO) mutant and a mucA KO mutant, respectively, whereas the evolved regulons were represented by the expression profiles of the corresponding NS mutants (the rpoN<sub>DK2</sub> and the mucA<sub>DK2</sub>algT<sub>DK2</sub> mutants). Comparisons between the native and the evolved regulons demonstrated that the evolved regulons were reduced to a subset of their respective WT regulons (Fig. 4A). Also, the magnitude of the expression changes on the target genes resulting from the evolved σ factors was reduced relative to response of the native σ factors (Fig. 4B). However, in some cases, the expression response from the evolved RpoN network was stronger or even opposite to that of native network (Fig. 4B, red points). In summary, the accumulated point mutations modified the activities of the σ factors, resulting in reduced or redirected regulatory capacity rather than loss of function.

**Adaptive Phenotypes of Evolved Regulatory Networks.** To investigate if the evolved regulatory networks impact on the adaptation of the DK2 lineage to the CF lung environment, we measured minimal inhibitory concentrations (MICs) of different CF relevant antibiotics against the collection of DK2 reconstruction mutants. Interestingly, we found that the Q mutant, compared with PAO1, was two- to fourfold less susceptible to tobramycin and ceftazidime, although neither the NS mutants nor the preceding mucA<sub>DK2</sub>algT<sub>DK2</sub>rpoN<sub>DK2</sub> triple mutant showed significantly changed susceptibility (Fig. 5). To test if the increased tolerance was related to epistatic effects of the lasR KO mutation in the quadruple background, and to rule out the possibility of second site mutations influencing the MIC determination, we reverted the Q mutant back to a triple mutant by allelic exchange of the mutated lasR allele with a WT copy. The resulting strain regained the fully susceptible phenotype, suggesting that the mechanism of increased tolerance toward these antibiotics was associated with the lasR mutation but highly dependent on the genetic configuration.

In concordance with the observation from the gene expression experiments, we find that the evolved alleles significantly impact on the motility of *P. aeruginosa*. Reduced motility is a common trait of clinical *P. aeruginosa* isolates from CF infections. Remarkably, the swimming and twitching of the Q mutant was reduced by ~87% and ~72% respectively, relative to PAO1. This reduction

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**Fig. 3.** Interactions between evolved regulatory networks. (A) Four-way Venn diagrams show the overlap between expression profiles of the NS mutants and the Q mutant. Expression profiles were divided into subprofiles of up- and down-regulated genes. Gray shading indicates the number of overlapping expression profiles (intersection degree), whereas numbers refer to the numbers of genes within each intersection. The proportion of false positives to be expected by chance within the expression profiles are indicated according to the intersection degree. Numbers in brackets indicate the total number of genes within each sub-expression profile. (B) Cluster I: cluster profiles of genes oppositely expressed as a result of counteracting effects of different mutations. Cluster II: cluster of genes being significantly expressed in two different NS mutants but weakly expressed in the Q mutant. Cluster III: cluster of genes being positively expressed in Q-mutant despite neutral or negative expression of in the NS mutants. Black line indicates the mean expression of the genes in each cluster. Dashed line indicates the mean (baseline) expression level of WT (PAO1).

**Fig. 4.** (A) Venn diagrams show size and overlap between native and evolved regulons of RpoN and AlgT, respectively, after correction for false positives. (B) Scatterplot shows regulatory response (fold changes) from the native and the evolved σ factors. Linear correlations are indicated by the squared value of *R*. Red data points indicate an opposite gene expression response between the native and the evolved network.
was stronger than expected from the additive contributions of the individual mutations (motility measurements of NS mutants; Table S1), suggesting that the quadruple configuration of mutations contribute to an epistatic reduction of motility in the Q mutant.

Evolution of Phenotypic Plasticity by Network Remodeling. One of the most common traits associated with clinical P. aeruginosa isolates from CF settings is constitutive overproduction of alginate resulting in a mucoid phenotype. From our reconstruction (Fig. 2A), we observed that the algT<sup>DK2</sup> mutation reverts the mucoid phenotype (resulting from the mucA<sup>DK2</sup> mutation) back to the nonmucoid phenotype—a phenotype also displayed by most DK2 isolates. However, the gene expression profile of the mucA<sup>DK2</sup>algT<sup>DK2</sup> mutant clearly showed elevated levels of a subset of the AlgT regulon (Fig. 4), which suggests that some activity is preserved in the AlgT<sup>DK2</sup> protein. From these observations, we hypothesized that specific environmental conditions could potentially generate an increased AlgT response from the evolved AlgT network. Indeed, we found that mutants containing the specific mucA<sup>DK2</sup>algT<sup>DK2</sup> configuration exhibited modified phenotypic plasticity and would transition from a nonmucoid to an alginate overproducing mucoid phenotype when exposed to CF-relevant stress conditions such as high osmolarity (0.2–0.5 M NaCl) and anaerobicity (Fig. 6). Importantly, this conditional phenotypic switch was uniquely linked to the mucA<sup>DK2</sup>algT<sup>DK2</sup> configuration and did not occur in PAO1 or a mucA<sup>DK2</sup>ΔAlgT mutant (Fig. S2 and Movie S1). This finding demonstrates that the normal relationship between environmental stimuli and phenotype has been reshaped by a specific algT<sup>DK2</sup> mutation in the DK2 lineage, and, more generally, the findings exemplify evolution of phenotypic plasticity by remodeling of an existing regulatory network.

Mutation in rpoD Results in Constitutive Mucoidy in DK2 Lineage. Conditionally induced alginate overproduction may be one of the traits that facilitated the evolutionary success of the DK2 lineage. The mutations conferring this trait were fixed in DK2 during the transition from its natural habitat to the CF environment and have been maintained in the DK2 lineage since 1973. Nevertheless we recently observed a sudden appearance of constitutively mucoid DK2 isolates in sputum samples from patient CF333 (two separate samples in 2007). This patient had been chronically infected with nonmucoid DK2 isolates for 16 y (from 1991 to 2007) before the emergence of the mucoid variants in 2007 (6, 9). Although the continued persistence of constitutive mucoid variant in later sputum samples from the patient suggest that perturbations in the airway environment resulted in selection of the mucoid phenotype, it is difficult to connect specific events to emergence of these variants. We measured the expression profile of the two constitutive mucoid DK2 isolates sampled in 2007 from CF333. Both isolates exhibited very similar but dramatically different gene expression patterns relative to what had been observed from its clonal predecessors in CF333 (i.e., the evolved expression phenotype of the post-1979 DK2 lineage; Fig. 7A).

To determine the underlying genetic changes that resulted in the constitutive mucoid phenotype, we sequenced the genomes of both mucoid 2007 isolates (Fig. 7B). One of the isolates contained a direct reversion of the one base missense mutation in algT<sup>DK2</sup>, resulting in a WT allele of algT. The other isolate maintained the algT<sup>DK2</sup> mutation but has instead acquired an in-frame 3-bp deletion (Glu507Δ) in rpoD encoding the primary σ factor RpoD (σ<sub>70</sub>; Fig. 7C). To test if this mutation (rpoD<sup>DK2</sup>) alone could facilitate constitutive overproduction of alginate, we inserted it in the genome of PAO1 by allelic replacement but did not observe any obvious phenotypic changes in the resulting strain. We subsequently moved the rpoD<sup>DK2</sup> allele into the mucA<sup>DK2</sup>algT<sup>DK2</sup> mutant genetic background. Strikingly, the resulting genotype (mucA<sup>DK2</sup>algT<sup>DK2</sup>rpoD<sup>DK2</sup>) displayed a constitutive highly mucoid phenotype similar to that of a PAO1 mucA mutant (Fig. 7D). These results demonstrate that the rpoD<sup>DK2</sup> mutation results in alginate overproduction only in association with a particular combination of mutations that arose decades earlier in the history of the DK2 lineage.

Discussion

Our results demonstrate that only a few genetic changes can have fundamental effects on the ecology, physiology, and fitness of microorganisms, and that evolutionary adaptation can involve remodeling of global gene regulatory networks rather than reorganization of the structural genes involved in specific traits. Reconstruction of four mutations in regulatory genes (mucA<sup>DK2</sup>, algT<sup>DK2</sup>, rpoN<sup>DK2</sup>, and lasR<sup>DK2</sup>) in PAO1 resulted in a phenotype shift toward the evolved phenotype found among

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Fig. 6. Phenotype switching in response to osmotic stress. Shown are changes in colony morphology of a mucA<sup>DK2</sup>algT<sup>DK2</sup> mutant and the WT (PAO1) during osmotic stress on agar plates (LB + 0.3 M NaCl). Both genotypes appear nonmucoid under normal growth conditions whereas only the mucA<sup>DK2</sup>algT<sup>DK2</sup> mutant becomes mucoid in response to osmotic stress. A time-lapse video is provided as Movie S1.

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Fig. 5. Epistatic effects cause increased tolerance toward tobramycin and ceftazidime. Shown are MIC values of tobramycin and ceftazidime for PAO1 and the PAO1-derived mutants. Double asterisks indicate significant differences (P < 0.01) in MIC relative to PAO1. ‘+ lasR’ indicates that the mutant was complemented (in cis) with a WT copy of lasR. Error bars represent SDs from three experiments.

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DK2 isolates sampled from patients after the transition of the DK2 lineage from its natural habitat to the CF environment. Although this strongly suggests that the particular combination of the four evolved regulator alleles was a major factor in determining the extraordinary ecological success (i.e., dissemination among a large group of patients during several decades) of the DK2 lineage, the appearance of a fifth regulatory mutation (in the \( \sigma \) gene encoding the primary \( \sigma \) factor) decades later in the evolutionary history of the DK2 lineage also shows that adaptation of the infectious bacteria involves ongoing evolutionary “tinkering” with regulatory network structures.

Epistatic interactions between mutations—which result in phenotypes that cannot be predicted from the individual mutations alone—play a fundamental role in the adaptation of the DK2 lineage and promote novel phenotypes. Epistatic interactions were observed in our gene expression measurements of the evolved regulatory networks, and we discovered that the \( lasR^{DK2} \) mutation confers reduced susceptibility to tobramycin and ceftazidime—two clinically relevant antibiotics—only in combination with the \( mucA^{DK2} \), \( algT^{DK2} \), and \( rpoD^{DK2} \) alleles. Also, similar to observations from laboratory evolution experiments in which epistatic interactions has been shown to restrain or open up certain evolutionary routes (10–12), we found that the recent appearance in patient CF333 of constitutive mucoid variants (as a result of the \( rpoD^{DK2} \) mutation) was contingent upon the \( mucA^{DK2} \) \( algT^{DK2} \) mutations that were fixed in the DK2 lineage earlier in time.

The airways of patients with CF constitute a complex and dynamic environment with fluctuating conditions as well as heterogeneous distributions of inhibitory and beneficial molecules (i.e., components of the immune system, antibiotics, nutrients, and other members of the CF microbiota) (13). Our detailing of the molecular effects of the regulatory mutations not only highlight the regulatory plasticity of biological networks but also demonstrate the evolutionary importance of mutations that reorganize the environmental responsivity of a network to function optimally in a dynamic environment such as CF airways: we found that a deletion in \( mucA^{DK2} \) resulted in constitutive production of the exopolysaccharide alginate (mucoid conversion) as expected from previous studies (14, 15), but that subsequent introduction of the \( algT^{DK2} \) allele altered the regulation of alginate biosynthesis and facilitated conditional expression of the mucoid phenotype only in response to certain CF-related environmental signals such as osmolarity and anaerobicity.

Mucoid conversion as a result of loss-of-function mutations in \( mucA \) is frequently observed in \( P. aeruginosa \) CF isolates (14, 15). Clinically, the presence of mucoid variants is associated with poor prognosis, deteriorating lung function, and increased tissue damage in patients (16), and marks the transition to the chronic stage of the infection (17). Mucoid variants are rarely if ever isolated from non-CF environments, and the mucoid phenotype (or other effects of de-repressed AlgT activity) represents an adaptive state of bacterial long-term persistence in the CF niche. Indeed, mucoid variants are better protected than nonmucoid cells against the inflammatory defense mechanisms of the host (18), and mucoid conversion is promoted by oxidative and osmotic stress conditions (19, 20). However, producing alginate in large amounts is metabolically demanding, and it has furthermore been demonstrated that mucoid conversion is associated with severe pleiotropic effects such as increased susceptibility to antibiotic used in the CF clinic (21). Thus, the evolved ability of the DK2 lineage to switch efficiently between the nonmucoid and the mucoid phenotype depending on the environmental context may ensure continued expression of the optimal phenotype solution.

The DK2 lineage is characterized by limited phenotypic diversification (Fig. 1), which contrasts the frequent observations of phenotypic heterogeneity in clonal \( P. aeruginosa \) populations within the same CF respiratory specimen (22–26) and evolutionary theory predicting enhanced diversified and specializing adaptation in spatially structured environments (27, 28). However, the evolution of the \( mucA^{DK2} \) \( algT^{DK2} \)-dependent conditional switch provides one possible explanation of this phenomenon, as the mutations remodel the normal phenotypic plasticity associated with the AlgT-controlled network and result in an optimized gene regulation that better fit the organism to many different challenges in the CF environment.

Nevertheless, other evolutionary solutions may be favored if the environmental conditions and selective pressures change significantly. The sudden emergence of two constitutive mucoid DK2 isolates in patient CF333 indeed suggests a significant change in the airway environment of the patient that favored selection of constitutive mucoid variants. Although the nature of these changes remains unknown, we note that the mucoid variants emerged after a period of noncompliance to antibiotic treatment.

The dynamics of mucoid/nonmucoid developments in the DK2 lineage point to a complex relationship between genomic changes, their interactions and history, and the ecological conditions. The mucoid phenotype developments are the result of point mutations in \( \sigma \) factors. We speculate that a possible molecular mechanism underlying the \( mucA^{DK2} \) \( algT^{DK2} \)-dependent conditional switching phenotype and the later \( rpoD^{DK2} \)-dependent constitutive mucoid conversion could be related to \( \sigma \) factor competition between AlgT and RpoD for binding to core RNA polymerase.

In conclusion, our results highlight the remodeling potential of regulatory networks and emphasize the importance of regulatory mutations and their interactions, which result in reorganization of cellular networks in such a way that some beneficial phenotypes are maintained whereas variations occur in others. More generally, the results also highlight the importance of investigating the exact polymorphisms selected by evolution instead of inferring the effects of regulatory mutations from information about gene function model systems or from experimentally induced loss-of-function mutations. Future investigations will reveal if other combinations of regulatory mutations can result in similar increased
adaptive performance in other *P. aeruginosa* lineages in relation to chronic CF infections.

**Materials and Methods**

**Bacterial Strains.** The bacterial strains and plasmids used in this study are described in Table S2. Construction of *P. aeruginosa* strains by allelic replacement is described in *SI Materials and Methods*. Briefly, the muck, algT, and rpoN alleles were amplified from genomic DNA from DK2 reference isolate CF333-2007. The *lsaR* locus was amplified from genomic DNA from the *lsaR* isolate H101212 (containing a KO mutation) and PA01. The *rpoD* locus was amplified from the mucoid isolate CF333-2007-prm. PCR fragments were cloned into the allelic replacement vector pN11 and verified by sequencing. The pN11-KO construct was made by insertion of a gentamicin resistance cassette into *rpoN* gene at the MfeI restriction site.Allelic replacement constructs were transferred into *P. aeruginosa* PA01 by triparental mating using the helper strain E. coli HB101/pRK600. 

**Phenotype Assays.** Motility assays, growth rate measurements, and determinations of MICs are described in *SI Materials and Methods*. For bacterial cultivation under different stress conditions, cells were grown on Luria–Bertiari (LB) plates supplemented with 0.2 to 0.4 M NaCl or 10% (v/v)ol sucrose and incubated for 24 to 48 h at 37 °C (osmolarity stress) or LB plates supplemented with 1% KNO3 and incubated in an anaerobic chamber (Coy Laboratories) at 37 °C (anaerobic stress).

**Genome Sequencing.** Genomic DNA was prepared from the DK2 isolate (CF333muarp and CF333munov) by using a Wizard Genomic DNA Purification Kit (Promega). Genomes were sequenced on an Illumina GAII generating 75-bp single-read reads by using a multiplexed protocol to an average coverage of 47-fold. Read mapping and mutation identification were done as previously described (6).

**Gene-Expression Profiling.** Transcriptomes were measured using Affymetrix PAO1 GeneChip for *P. aeruginosa*. All *P. aeruginosa* strains were grown aerobically at 37 °C in LB medium starting from an OD600 of 0.01 and harvested at an OD600 of 0.5. Harvested cells were mixed immediately with RNAprotect Bacteria Reagent (Qiagen) and stored at −80 °C. RNA extraction, cDNA labeling, and hybridization were done as previously described (6). Three biological replicates were made for each bacterial strain tested. Data analysis was performed by using R (29) with Bioconductor packages (30). Raw CEL files were processed using the robust multichip average (rma) algorithm available in the affy package (31), with steps including background correction, quantile normalization, and summarization by the median polish approach (32). The log2-scaled data from robust multichip average were used in statistical testing (*SI Materials and Methods*).

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