Global reprogramming of virulence and antibiotic resistance in *Pseudomonas aeruginosa* by a single nucleotide polymorphism in elongation factor, *fusA1*

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Clinical isolates of the opportunistic pathogen *Pseudomonas aeruginosa* from patients with cystic fibrosis (CF) frequently contain mutations in the gene encoding an elongation factor, *FusA1*. Recent work has shown that *fusA1* mutants often display elevated aminoglycoside resistance due to increased expression of the efflux pump, MexXY. However, we wondered whether these mutants might also be affected in other virulence-associated phenotypes. Here, we isolated a spontaneous gentamicin-resistant *fusA1* mutant (FusA1P443L) in which *mexXY* expression was increased. Proteomic and transcriptomic analyses revealed that the *fusA1* mutant also exhibited discrete changes in the expression of key pathogenicity-associated genes. Most notably, the *fusA1* mutant displayed greatly increased expression of the Type III secretion system (T3SS), widely considered to be the most potent virulence factor in the *P. aeruginosa* arsenal, and also elevated expression of the Type VI (T6) secretion machinery. This was unexpected because expression of the T3SS is usually reciprocally coordinated with T6 secretion system expression. The *fusA1* mutant also displayed elevated exopolysaccharide production, dysregulated siderophore production, elevated ribosome synthesis, and transcriptomic signatures indicative of translational stress. Each of these phenotypes (and almost all of the transcriptomic and proteomic changes associated with the *fusA1* mutation) were restored to levels comparable with that in the progenitor strain by expression of the WT *fusA1* gene in trans, indicating that the mutant gene is recessive. Our data show that in addition to elevating antibiotic resistance through *mexXY* expression (and also additional contributory resistance mechanisms), mutations in *fusA1* can lead to highly selective dysregulation of virulence gene expression.

Due to its high intrinsic resistance to antibiotics and aggressiveness, *Pseudomonas aeruginosa* holds the dubious accolade of consistently occupying a “top ten” slot on lists of clinical threats across the globe. Indeed, the World Health Organization recently classified it as a top priority pathogen for which the development of new antimicrobial interventions is critical. This opportunistic, Gram-negative bacterium is ubiquitous and exhibits a particular predilection for the built environment, making encounters with the human populace commonplace. *P. aeruginosa* is frequently isolated from burn wounds, the respiratory tract, and the urinary tract and is the leading cause of morbidity and mortality in people with cystic fibrosis (CF) (1–3). CF is a genetic disease characterized by defective targeting or activity of the cystic fibrosis transmembrane conductance regulator. Although it is a multisystem disease affecting many organs, the most obvious manifestations of CF are associated with the respiratory tract. Here, defective mucociliary clearance causes an accumulation of thick mucus plugs within the airways. Such oxygen-limited environments provide the perfect niche for *P. aeruginosa* to thrive (4, 5).

Chronic *P. aeruginosa* infection of the CF lung is associated with the transition from an active, motile lifestyle to a sessile, biofilm-like mode of growth. These are bacterial communities embedded within a self-produced extracellular polymeric matrix, composed of mannose-rich polysaccharides, extracellular DNA, and proteins (6). This matrix confers a level of protection against antibiotics and the host immune system (7). Biofilm formation is also associated with increased expression of the Type VI (T6) secretion machinery. The function of the *P. aeruginosa* T6 secretion system has become clearer in the last decade; it appears to play a role in killing other bacterial species (or even “non-self” *P. aeruginosa* strains) (8, 9), especially in tightly packed biofilms where competition for the same resources is rife. Conversely, “free-swimming” planktonic cells predominate in acute infection scenarios. Here, virulence factors and motility are up-regulated, and pathogenicity is enhanced (10). Perhaps the most potent *P. aeruginosa* virulence determinant is the Type III (T3) secretion system, which mediates the translocation of cytotoxic effector proteins directly into the cytoplasm of neighboring host cells. These effectors subvert the function of the recipient cells, typically by disrupting the cytoskeleton, promoting cell rounding and apoptosis, and therefore assist immune evasion through preventing phagocytosis by host innate immune cells (11, 12). Both the T3 and T6 secretion systems are contact-triggered injectisomes; however, they are structurally and mechanistically distinct, as are their targets, and the expression of these two systems appears to be inversely correlated (13).

In previous work, we showed that the rapidly growing planktonic cells associated with increased virulence factor production display markedly up-regulated expression of the machinery required for macromolecular synthesis, especially proteins involved in translation (14). Translation comprises four main steps; initiation, elongation, termination, and recycling. The ribosome-associated protein, elongation factor G (EF-G, encoded by *fusA1* and *fusA2* in *P. aeruginosa*), is essential for
two of these steps: “elongation” and “recycling” (15). During elongation, EF-G catalyzes the translocation of charged tRNA from the A-site to the P-site and from the P-site to the E-site of the large ribosomal subunit. This is coupled with movement of the ribosome along the mRNA being translated. EF-G is comprised of five domains; domains I and II mediate GTP binding and hydrolysis, and domains III, IV, and V dock to the A-site of the ribosome. The tip of domain IV interacts with the mRNA and promotes tRNA translocation (16). This involves large structural rearrangements as the EF-G domains swivel relative to one another (17, 18). The translocation process is repeated until a stop codon is encountered and release factors catalyze hydrolysis of the peptidyl-tRNA bond, thereby liberating the newly synthesized polypeptide. EF-G then coordinates with ribosome-recycling factor (a structural mimic of tRNA) to promote disassembly of the ribosomal subunits (16, 19). Aminoglycoside antibiotics can disrupt both the elongation and recycling steps, thereby leading to a decrease in the overall number of ribosomes available.

Whole-genome sequence analyses have revealed that fusA1 is a hotspot for accruing mutations in P. aeruginosa isolates from patients with CF (20). These fusA1 mutants often display increased resistance to aminoglycoside antibiotics. However, little more is known about the phenotypic consequences of such fusA1 mutations on the wider physiology of the cell. In this study, we show that a spontaneous SNP in P. aeruginosa fusA1 gives rise to discrete but large-magnitude changes in key pathophysiological processes. For example, the strain containing the mutated fusA1 allele displayed selective up-regulation of genes encoding the T3 secretion system (T3SS) apparatus, the T6 secretion system (T6SS) apparatus, exopolysaccharide biosynthesis genes, and a multidrug efflux system. These findings suggest a hitherto unexpected subtlety in the chain of events linking transcription, translation, and virulence/antibiotic sensitivity in this organism.

Results

An SNP in fusA1 causes decreased expression of the biofilm-associated protein, CdrA

At the outset of this investigation, we sought to identify potential regulators of biofilm formation in P. aeruginosa. To do this, we made a stable chromosomal reporter construct in which the promoter of the cdrAB operon (PcdrAB) was fused to a promoter-less lacZ ORF. CdrA is a biofilm-associated extracellular matrix adhesion and is known to be primarily expressed in conditions that favor biofilm formation. The construct was integrated at a neutral site in the PAO1 chromosome using the mini-CTX system (21). The resulting PcdrAB::lacZ reporter strain, hereafter referred to as EMC0, yielded vivid blue colonies when grown on M9 minimal medium agar plates containing X-Gal and glucose, but far paler colonies on medium containing X-Gal and glycerol. This suggested that PcdrAB is activated during colony growth on glucose. To identify genes that might impinge upon transcription from the cdrAB promoter, we mutagenized EMC0 by introducing the TnModOGm plasposon (22). The resulting mutants were selected on plates containing gentamicin (to select for likely Tn insertion mutants) and X-Gal + glucose (to establish whether any of the mutants were affected in transcription from PcdrAB). Around 10,000 gentamicin-resistant mutants were screened in all. Several of these yielded a paler pigmentation than EMC0 when grown on X-Gal/glucose plates, indicating a reduction in transcription from PcdrAB (Fig. 1A). Further analysis of one of these isolates (hereafter denoted EMC1) in M9-glucose liquid cultures confirmed the diminished β-gal production. This also indicated that EMC1 had a minor growth defect in this medium (Fig. 1A). We therefore measured growth and β-gal production in a rich medium, LB. To our surprise, EMC1 exhibited an even greater growth defect in this medium (Fig. 1B).

Attempts to identify the insertion site of the plasposon in EMC1 using conventional approaches (including “random-primed” PCR-based amplification of the regions flanking the plasposon, or “cloning out” of the plasposon as described previously (22)) failed. We therefore used whole-genome sequencing (WGS) of EMC1 (and, as a control, also of the EMC0 progenitor strain) to identify the plasposon insertion site. Remarkably, and despite the robust GmR phenotype of the strain (Table 1), we found that EMC1 did not contain a plasposon insertion. It did, however, contain a C→T transition at position 4,770,363 in the genome (Fig. S1). This SNP was located in the fusA1 ORF and resulted in a proline-to-leucine substitution at position 443 in the protein. The SNP was confirmed by PCR amplification of the gene followed by Sanger sequencing of the PCR product. WGS also revealed a selection of additional potential SNPs in EMC1, but these were all subsequently found by PCR/Sanger sequencing to be false positive SNP calls arising from the WGS technology. We conclude that the only difference between EMC0 and EMC1 is the SNP in fusA1.

The gentamicin resistance of EMC1 was heritable but could be somewhat unstable. In one experiment, progressive subculturing (via 1:100 dilution of the culture into fresh medium every 24 h) of EMC1 in M9-glucose minimal medium lacking gentamicin gave rise to gentamicin-sensitive (GmS) derivatives. After each 24-h round of subculturing, aliquots were removed, serially diluted, and plated onto nonselective M9-glucose agar. A selection of 40 colonies from these plates were then retested for gentamicin resistance by plating onto M9-glucose + Gm. Following the first round of subculturing (24-h growth), none of the 40 tested colonies were GmS. However, after the second round of subculturing (a cumulative 48 h of growth) 1 of 40 colonies tested was gentamicin-sensitive, rising to 5 of 40 colonies after the third 24-h round of subculturing.

PCR amplification and sequencing of the fusA1 gene in these GmS derivatives revealed that they all still contained the fusA1P443L mutation. These data suggest that whatever gives rise to the GmR phenotype in EMC1 can be overridden by secondary mutations elsewhere. However, these bypass mutations do not arise with high frequency, and even when they did, they took time to spread through the culture (with only 1 in 8 of the isolates sampled being GmR after 72 h of growth). Indeed, subsequent independent repeats of the experiment yielded no further GmS isolates upon subculturing EMC1 in the absence of gentamicin.
FusA1 contains 6 tryptophan residues. Protein Trp fluorescence is exquisitely sensitive to the microenvironment of each Trp residue and, as such, can be a sensitive reporter of protein conformation. These analyses indicated that purified FusA1P443L had a lower quantum yield at the Trp emission $\lambda_{\text{max}}$ (332 nm) compared with the WT protein (Fig. 2B). This indicates that one or more Trp residues in the mutant protein are likely to exhibit altered solvent accessibility compared with the WT protein, possibly due to conformational differences. One of the more widely used web-based algorithms, mCSM (25), predicted that the P443L substitution should destabilize FusA1 by 0.267 kcal/mol. Consistent with this, purified FusA1P443L had a lower melting temperature than the WT protein (Fig. 2C). Taken together, these data indicate that the P443L substitution likely alters the conformation, stability, or dynamics of FusA1.

Table 1

| Antibiotic          | Mode of action                                      | EMC0 MIC $\mu g/ml$ | EMC1 MIC $\mu g/ml$ |
|---------------------|-----------------------------------------------------|---------------------|---------------------|
| Gentamicin          | Binds 30S ribosome and disrupts reading of tRNA     | 2                   | 10                  |
| Kanamycin           | Binds 30S ribosome and disrupts reading of tRNA     | 1500                | $>2000$             |
| Tetracycline        | Binds 30S ribosome and inhibits binding of tRNA     | 20                  | 20                  |
| Chloramphenicol     | Binds 50S ribosome and inhibits peptide bond formation | 250                | 250                |
| Rifampicin          | Inhibits RNA polymerase                             | 100                 | 100                 |
| Fusidic acid        | Inhibits elongation factor G                         | 1750                | 1750                |

The proline $\rightarrow$ leucine substitution at position 443 in FusA1 affects protein stability and conformation

FusA1 encodes one of the two paralogous EF-G proteins in *P. aeruginosa* and plays a pivotal role in protein synthesis and ribosomal recycling (23). The amino acid sequence of the two paralogues (denoted fusA1 (PA4266) and fusA2 (PA2071)) is highly conserved, with a shared identity of 84%. EF-G1B, encoded by fusA2, is thought to have greater involvement in elongation and polypeptide synthesis (23). By contrast, EF-G1A (encoded by fusA1) has a more dominant role in ribosomal recycling and association with ribosomal recycling factors (23). FusA1 is a conformationally flexible multidomain protein (24), and proline 443 sits in close proximity to the crucial GTPase “switch” regions (Fig. 2A). The switch regions play an important role in directing GDP-GTP exchange, raising the question of whether the P443L substitution might affect the conformation of the protein. To test this, we measured the intrinsic Trp fluorescence profile of purified WT FusA1 and FusA1P443L. FusA1 contains 6 tryptophan residues. Protein Trp fluorescence is exquisitely sensitive to the microenvironment of each Trp residue and, as such, can be a sensitive reporter of protein conformation. These analyses indicated that purified FusA1P443L had a lower quantum yield at the Trp emission $\lambda_{\text{max}}$ (332 nm) compared with the WT protein (Fig. 2B). This indicates that one or more Trp residues in the mutant protein are likely to exhibit altered solvent accessibility compared with the WT protein, possibly due to conformational differences. One of the more
EMC1 compared with EMC0, both on plate assays (Fig. 3A) and in liquid culture (Fig. S3). Expression of WT fusA1 in trans in EMC1 led to a decrease in exopolysaccharide production (compared with EMC1 containing the empty vector control), whereas expression of fusA1P443L enhanced exopolysaccharide synthesis. Exopolysaccharides comprise the extracellular matrix, which “glues together” cells in a biofilm. Interestingly in this regard, the increased exopolysaccharide production in EMC1 was not accompanied by an increase in its biofilm-forming ability compared with EMC0 (data not shown). Exopolysaccharide production is often inversely correlated with expression of the T3 secretion machinery. We therefore examined whether the P443L substitution in FusA1 impacted T3 secretion. To our surprise, cultures of EMC1 overexpressed the T3SS protein, PcrV (Fig. 3B). This increased expression was due to increased transcription of the pcrV-encoding operon, because RT-PCR analyses indicated that the amount of mRNA encoding pcrV was also increased in EMC1 (Fig. 3B and

Figure 2. Structural impact of the P443L substitution on FusA1. A, location of the P443L substitution on the FusA1 structure. Proline 443 is highlighted in red and Leucine 443 is highlighted in blue. B, the tryptophan fluorescence emission spectrum of 0.8 μM purified WT FusA1 (blue line) and FusA1P443L (green line) suggests that one or more Trp residues resides in an altered microenvironment in FusA1P443L. C, thermal shift data indicate that the WT protein (top) has a higher melting temperature than the FusA1P443L protein (bottom). This suggests that the P443L substitution decreases the thermal stability of the protein. Each melting curve was measured in triplicate.

Figure 3. The fusA1P443L mutation has pleiotropic effects on the cell. A, the twitching, swimming, gelatinase, and exopolysaccharide phenotypes of EMC0 and EMC1 (as indicated) containing pUCP20 (empty vector control), pUCP20 encoding WT fusA1, or pUCP20 encoding fusA1P443L. Increased exopolysaccharide production is indicated by more intense staining of the colony with Congo Red. Expression of the fusA1 mutant and WT ORFs cloned into pUCP20 was driven from the plasmid-encoded lac promoter. B, expression of PcrV protein (top) and pcrV-encoding mRNA (bottom) is increased in EMC1 and complemented by expression of WT fusA1 in trans. Representative results are shown. For biological and technical replicates and controls (PCR amplification from non-reverse-transcribed RNA samples, PCR amplification from gDNA, sizing markers for DNA gels and Western blots, etc.), see Fig. S4.
Global impact of fusA1 mutation

Table 2
List of the top 20 proteins (based on log2 FC) modulated in EMC1 versus EMC0
At the top are shown up-regulated proteins, rank-ordered by FC abundance. At the bottom are shown down-regulated proteins, rank-ordered by FC abundance

| Up-regulated proteins | Locus tag | Protein function | EMC1 versus EMC0 |
|-----------------------|-----------|------------------|------------------|
| 1 PscL PA1725         |            | Type III secretion export protein | 5.873 0.000 |
| 2 PopN PA1698         |            | Type III secretion outer membrane protein | 4.187 0.001 |
| 3 PscP PA1695         |            | Type III secretion translocation protein | 4.178 0.000 |
| 4 PscD PA1709         |            | Hypothetical protein | 4.004 0.002 |
| 5 PopD PA1325         |            | Type III secretion outer membrane protein | 3.895 0.000 |
| 6 PcrV PA2191         |            | Adenylate cyclase | 3.266 0.000 |
| 7 PcrH PA1707         |            | Regulatory protein | 3.262 0.000 |
| 8 PcrG PA1705         |            | Type III secretion regulator | 3.211 0.000 |
| 9 PcrB PA1708         |            | Type III translocator protein | 3.151 0.000 |
| 10 PcrH PA2044        |            | Exoenzyme T | 2.836 0.000 |
| 11 PcrD PA1245        |            | Metalloproteinase | 2.798 0.001 |
| 12 AprX PA1249        |            | Alkaline metalloprotease | 2.755 0.007 |
| 13 PelA PA3064        |            | Exopolysaccharide biosynthesis | 2.725 0.000 |
| 14 PscF PA1719        |            | Hypothetical protein | 2.688 0.001 |
| 15 PscG PA1707        |            | R-type pyocin, related to P2 phage | 2.613 0.000 |
| 16 PscH PA1707        |            | Hypothetical protein | 2.519 0.002 |
| 17 PscI PA1725        |            | Type III export protein | 2.507 0.000 |

| Down-regulated proteins | Locus tag | Protein function | EMC1 versus EMC0 |
|------------------------|-----------|------------------|------------------|
| 1 ExaB PA1983          |            | Ethanol oxidation | 3.154 0.000 |
| 2 PscL PA5086          |            | Predicted T6SS lipase immunity protein | 3.088 0.000 |
| 3 ExaB PA2565          |            | Hypothetical protein | 2.962 0.002 |
| 4 PscP PA3875          |            | Hypothetical protein | 2.955 0.001 |
| 5 PscQ PA3841          |            | Hypothetical protein | 2.947 0.000 |
| 6 PscR PA2070          |            | Hypothetical protein | 2.807 0.001 |
| 7 PscS PA2134          |            | Hypothetical protein | 2.768 0.006 |
| 8 PscT PA3576          |            | Hypothetical protein | 2.548 0.004 |
| 9 ExoS PA3063          |            | Hypothetical protein | 2.533 0.001 |
| 10 PscU PA2505         |            | Hypothetical protein | 2.471 0.000 |
| 11 PscV PA1467         |            | Hypothetical protein | 2.467 0.002 |
| 12 PscW PA3539         |            | Hypothetical protein | 2.409 0.001 |
| 13 PscX PA4063         |            | Hypothetical protein | 2.366 0.000 |
| 14 PscY PA3318         |            | Hypothetical protein | 2.300 0.067 |
| 15 PscZ PA2781         |            | Hypothetical protein | 2.291 0.001 |
| 16 PscA PA4063         |            | Hypothetical protein | 2.278 0.000 |
| 17 PscB PA0314         |            | Flagellar rotor protein | 2.259 0.001 |
| 18 PscC PA5330         |            | Hypothetical protein | 2.240 0.001 |
| 19 PscD PA5088         |            | Predicted T6SS lipase immunity protein | 2.205 0.000 |

Fig. S4). This increased expression of PcrV could be reversed by supplying WT fusA1 in trans.

These observations led us to test whether previously reported GmR fusA1 point mutants also displayed increased PcrV expression. A selection of GmR fusA1 mutants (all in a PAO1 genetic background) recently reported by Bolard et al. (20) were grown (alongside the progenitor PAO1 strain from that study) in M9-glucose, and the corresponding cell extracts were probed after SDS-PAGE resolution with anti-PcrV antibodies. None of these mutants displayed the robust PcrV expression associated with EMC1 (Fig. S5). This suggests that the GmR phenotype conferred by these other mutated fusA1 proteins is not linked with PcrV expression.

Global consequences of the fusA1P443L mutation on the proteome

Given that multiple phenotypes were affected by the fusA1P443L mutation in EMC1 and that these phenotypes were not all modulated in the manner expected from previous studies (e.g. exopolysaccharide production and T3 secretion both being up-regulated instead of inversely regulated), this suggested that the P443L mutation may lead to global dysregulation in EMC1. To investigate this further, cultures of EMC0, EMC1, and EMC1 complemented with WT fusA1 expressed from pUCP20 in trans (hereafter EMC1*) were grown to late exponential phase in M9 minimal medium + glucose and were prepared for iTRAQ-based proteomic analysis. To establish whether any of the observed changes in protein profile were also underpinned by transcriptional changes, samples were also harvested for RNA sequencing (from cultures grown in the same conditions).

The proteomic analysis resolved 3506 proteins (of a total of 5570 predicted ORFs encoded by P. aeruginosa PAO1). Principal components analysis of the data revealed that the proteome of EMC1 was distinct from that of EMC0 and that the proteomic changes giving rise to this segregation could be largely reversed by expression of WT fusA1 in trans in EMC1* (Fig. S6 (A–C) and Tables S1 and S2). Proteins were considered to be significantly modulated if they exhibited a log2-fold change (FC) > 1 (or < –1, if down-regulated) with a false discovery rate (FDR)-adjusted p value of ≤0.01. Based on these criteria, 128 proteins were up-regulated in EMC1 compared with EMC0, and 166 proteins were down-regulated. The 20 most highly up-regulated proteins are shown in Table 2. Remarkably,
and consistent with the earlier phenotypic analyses, over half (12 of 20) of these proteins were involved in T3 secretion. Similarly, and also consistent with our earlier observations, PelA, involved in the biosynthesis of exopolysaccharide, was also up-regulated. A list of the top 20 down-regulated proteins is shown in Table 2. The situation here is more ambiguous, because the majority (13 of 20) of these proteins currently have no assigned function.

To obtain a more functional overview of the data, the 128 statistically significantly up-regulated proteins in EMC1 were analyzed using STRING to identify clusters of associated proteins. STRING is a database of known and predicted physical and functional protein-protein interactions (26). Inspection of the STRING output revealed that the modulated proteins fell into several distinct functional clusters involved in a variety of pathophysiological processes. The most obvious cluster comprised proteins associated with the T3SS (Fig. 4). The likely driver behind this was the ~2-fold up-regulation of ExsA, which is the master regulator (activator) of T3SS expression (27). Also consistent with the phenotypic data, a cluster of proteins (PelA, PelF, and PslG) involved in exopolysaccharide biosynthesis were up-regulated in EMC1. The data also revealed a probable explanation for the enhanced gentamicin resistance of EMC1; ArmZ, a major activator of the mexXY aminoglycoside efflux pump, was up-regulated, as was expression of MexXY (28, 29). Unexpectedly, we also noted that a selection of proteins involved in biosynthesis of the siderophore, pyochelin, were significantly up-regulated. This was somewhat surprising because (i) proteins associated with the biosynthesis of the other major siderophore in P. aeruginosa, pyoverdine, were unaffected; (ii) the abundance of iron-uptake master regulators, Fur and PvdS, as well as the pyochelin-specific regulator PchR were unchanged in EMC1 compared with EMC0; and (iii) phenotypic analyses revealed that cultures of EMC1 produce less secreted siderophore(s) than EMC0 or EMC1* (Fig. S7). It therefore seems that in EMC1, the biosynthetic pathway for pyochelin is expressed, but the siderophore is not secreted. This dysregulation of iron homeostasis in EMC1 may have additional consequences. A link between iron availability and T3 secretion has been documented in several bacterial genera, including Bordetella, Salmonella, Shigella, Edwardsiella, Vibrio, and Yersinia (30–35). To investigate further

Figure 4. STRING-based analysis of up-regulated proteins in EMC1. The indicated clusters are discussed under “Results.”
whether iron availability impacts upon expression of the T3SS in EMC1, we examined whether supplementation of the growth medium with additional iron had any effect on PcrV expression. The additional iron had little effect on expression of PcrV in EMC0 or EMC1*. However, the addition of excess iron to the EM1 cultures led to increased PcrV expression (Fig. S7). It is therefore possible that the dysregulation of iron homeostasis in EMC1 is another factor that impacts expression of the T3SS.

A number of bacteriophage-like R2-type pyocin gene products were also up-regulated. R-type pyocins cause membrane depolarization and inhibit active transport in closely related species to reduce bacterial competition (36). Another obvious functionally related grouping was comprised of proteins associated with sulfur metabolism (including CysA, CysD, SsuD, TauA, and TauB). However, the most notable cluster of up-regulated metabolic proteins were those involved in polyamine catabolism (PauA4, PauA5, PauB1, PauB3, and PauB4). The genes encoding these proteins are strongly induced by the polyamines, putrescine and spermidine, and are distributed across the chromosome (37). These gene products encode probable \( \gamma \)-glutamylpolyamine synthases involved in converting putrescine into \( \gamma \)-aminobutyric acid, and their collective up-regulation indicates that putrescine and/or spermidine may be more abundant in cultures of EMC1. Consistent with this notion, spermidine is known to also increase the expression of the T3SS proteins (38).

We also carried out a STRING-based analysis of the proteins that were down-regulated in EMC1. Fewer clusters were apparent compared with the up-regulated STRING map. This likely reflects the large number of uncharacterized proteins in the data set; with little information to functionally link these proteins, clusters will inevitably be more sparsely populated. However, some patterns were apparent. From the STRING map (Fig. S8), a small cluster comprising ExaB, nitrite reductase (NirS), and azurin (Azu) is discernable. ExaB is of interest because it was the protein with the greatest FC (Table 2). ExaB is a cytochrome \( \epsilon_{550} \) involved in the breakdown of ethanol to aldehyde (39). Azurin transfers electrons from another \( c \)-type cytochrome, cytochrome \( \epsilon_{551} \), to cytochrome oxidase. Cytochrome \( \epsilon_{551} \) is also the electron donor of nitrite reductase (NirS) and plays a role in dissimilative denitrification (40). These findings suggest that elements of the bacterial electron transport chain may be affected in EMC1. The ExaB/NirS/Azu cluster was linked to an adjacent group of proteins with roles in disulfide bond formation (DsbA, DsbD2, a probable GSH peroxidase (PA1287), TrxA, and three other Trx-like proteins (PA0941, PA3664, and PA0950)) and protein folding (PpiA and PpiC2). These proteins are closely linked to the electron transport chain and cellular redox status.

Several other highly down-regulated proteins were linked to the T6SS. PA5086 and PA5088 are predicted to encode T6SS lipase immunity proteins, protecting the producing cell from lipase-inflicted self-harm (41). PA5088 is operonic with an encoded T6SS phospholipase D effector (tleBS) and with vgrG5, although neither of these proteins were detected in our experiments. VgrG proteins are secreted by the T6SS to form complexes that perforate host cell membranes, and the genomic position of vgrG5 adjacent to PA5088 makes it likely that its expression was also reduced. The down-regulation of T6SS-associated proteins observed here is consistent with the known inverse regulation of T6 and T3 secretion (13). Another discernible cluster of proteins was related to cell motility and chemotaxis, including CheR1, PA0177, PctC, PA1464, and the twitching motility protein, PiiH. The flagellar motor protein, FlfY, and an uncharacterized protein, PA2781, were also down-regulated. PA2781 is likely operonic with PA2780 (bswR), a bacterial swarming regulator (undetected in the proteomic analysis) and is predicted to interact with FlgB, a structural component of the bacterial flagellum. This suggests that PA2781 may also affect flagella-mediated motility. Collectively, these observations are consistent with the decreased twitching and swimming motility associated with EMC1 (Fig. 3).

**Global impact of fusA1 mutation**

Given the role of FusA1 in translation and given the apparently very selective consequences of the \( fusA1^{P443L} \) mutation on the proteome, we wondered whether these changes might also be reflected at a transcriptomic level. To address this possibility further, we carried out an RNA-Seq analysis of the mRNA expression profiles in EMC1 and EMC1* compared with EMCO. Of the 5678 predicted RNA-encoding genes in \( P. aeruginosa \) PAO1, 5628 were represented in the RNA-Seq data set. This suggests that most ORFs are expressed at detectable levels in the conditions tested. Of these expressed genes, 657 were significantly \( (p < 0.01) \) up-regulated \( (FC > 1) \), whereas 374 were significantly down-regulated \( (FC > -1) \) when comparing EMCO with EMC1. Far fewer transcripts were significantly modulated when comparing EMCO with EMC1*. Consistent with the proteomic analysis, \( exsA \) expression was up-regulated by around 3-fold and is most likely to be a major factor driving the observed global up-regulation of the T3SS transcripts. It is worth noting that the transcriptional regulator PsrA is thought to be required for full activation of the \( exsCEBA \) operon (42). However, \( psrA \) transcripts exhibited a 2-fold reduction in expression, suggesting that PsrA may not be a major requirement for T3 secretion in all conditions and that
low levels of psvA transcription do not necessarily prevent expression of the exsCEBA operon. Other distinct clusters predicted by STRING included a group of ribosome-associated proteins and, surprisingly, also a robust cluster of T6SS-associated transcripts (mostly from Hcp secretion island I (HSI-I)). Indeed, the transcript encoding the HSI-I needle protein, Hcp1, was up-regulated 4-fold, and many other T6-associated transcripts were up-regulated >4-fold. This was unexpected because the T3SS and T6SS are reciprocally regulated in most conditions, and several T6-associated proteins were down-regulated in the proteomic analyses. Given that we previously noticed that Gm^S^ mutants can potentially arise in the EMC1 culture, elevated expression of the HSI-I T6SS protein, PcrV, should only be present in the cultures grown without Gm. We then blotted the cell extracts from each culture for the HSI-I needle protein (Hcp1) and also for a T3S system protein (PcrV). As shown in Fig. S10, PcrV expression was basal in the PAO1 and EMC0 cultures, irrespective of the presence of sub-MIC~Gm~. However, PcrV expression was robust in both the presence and the absence of Gm in the EMC1 cultures. By contrast, Hcp1 expression was moderate to high in the PAO1 and EMC0 cultures grown without Gm but was low in the EMC1 culture grown without Gm. We conclude that in conditions that may potentially allow for the growth of Gm^S^ bypass mutants in the absence of Gm, a single colony from each plate was then used to inoculate M9-glucose liquid medium containing either no Gm (PAO1, EMC0, and EMC1) or 1 μg ml^{-1} Gm (0.5 × MIC_{Gm}) in the case of EMC0 or 5 μg ml^{-1} Gm (0.5 × MIC_{Gm}) in the case of EMC1. In this setup, the Gm^S^ EMC1 starter culture should contain no Gm^S^ cells, and if these arise at all, they should only be present in the cultures grown without Gm. We then tested the cell extracts from each culture for the HSI-I needle protein (Hcp1) and also for a T3S system protein (PcrV).
previous reports, increased expression of the T3SS (as assessed by PcrV abundance) in EMC1 correlates with decreased expression of the T6SS (as assessed by Hcp1 abundance). We also noted that exposure to sub-MIC aminoglycoside (here, Gm) strongly induced expression of the T6SS in EMC1 (as reported previously by Jones et al. (43)), although in the EMC1 background, this was not accompanied by a decrease in PcrV expression. Although the list of most highly up-regulated transcripts was dominated by the T3SS genes, the two genes with the highest modulation in EMC1 were an operonic pair, PA1325 and PA1326 (Table 3). PA1326 (ilvA2) encodes a threonine dehydratase involved in glycine, serine, and threonine metabolism, and the adjacent gene, PA1325, is uncharacterized. Indeed, a relatively large number of genes linked to amino acid metabolism were found to be up-regulated in this region of the genome. For example, the nearby ggt (PA1338) gene encodes a γ-glutamyltranspeptidase involved in GSH catabolism (up-regulated in EMC1 2.6-fold). Adjacent to ggt is another set of up-regulated genes encoding an ABC transport system for glutamate and aspartate (aatP (PA1339, ↑4.0-fold), aatM (PA1340, ↑4.5-fold), aatQ (PA1341, ↑6.1-fold), and aatI (PA1342, ↑3.7-fold)). Another up-regulated gene was PA3965 (↑2.4-fold), encoding an AsnC-type transcriptional regulator that, although uncharacterized, is one of just two Lrp homologs encoded by PAO1. In P. aeruginosa, Lrp regulates the expression of genes involved in amino acid biosynthesis and catabolism (44). We also noted that transcripts from an uncharacterized gene cluster (PA3327–PA3336) encoding a nonribosomal peptide synthase were up-regulated. The product of the nonribosomal peptide synthase in the cluster (PA3327, ↑6.6-fold)
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was predicted by antiSMASH (45) to generate a dipeptide core based around the condensation of serine and proline. Another large cluster (comprising 62 up-regulated transcripts in all) encoded ribosomal proteins and translation factors. Of these, 20 encoded 30S subunit proteins, and 26 encoded 50S subunit proteins. Elongation factor Tu, initiation factor-2 and peptide chain release factors, were also up-regulated. One possible reason for the overall up-regulation of ribosomal transcripts in EMC1 came from the earlier proteomic analysis, which revealed increased abundance of SmpB. This is an RNA-binding protein that is induced upon ribosomal stalling. The increased expression of SmpB indicates that the mutated FusA1 likely leads to decreased translation efficiency and is compensated for by an increase in the overall expression of the translation machinery. Several genes involved in RNA processing were also up-regulated, including the RNA 3’-terminal phosphate cyclase (rtcA, ↑5.2-fold) and RNA ligase (rtcB, ↑9.3-fold).

A large number of transcripts were also down-regulated in EMC1, although generally, the magnitude of modulation was low (Table 3). A STRING analysis of the 200 most highly down-regulated transcripts is shown in Fig. S11. Rather few distinct clusters were apparent; again, this is likely due to the predominance of transcripts from uncharacterized genes in the data set. However, a few of the identified genes are worthy of comment. First, the small untranslated RNA, rsmY (PA0527.1) was down-regulated 3.8-fold. The rsmY-binding partner and antagonist, RsmA, was also down-regulated (↓5.7-fold). These are potentially important observations because excess “free” (i.e. unquenched, by rsmY) RsmA increases expression of the T3SS genes and depresses expression of the T6SS/exopolysaccharide genes (8). The expression of rsmY is thought to be regulated by the Gac signaling pathway. However, none of the other Gac/Rsm pathway-encoding transcripts (ladS, retS, gacA, gacS) were modulated in EMC1, nor was rsmZ (another untranslated small RNA thought to act in a similar manner to rsmY). If current models are correct, a stoichiometric imbalance of rsmY/RsmA levels should impinge reciprocally on T6SS expression and extracellular polysaccharide production (on the one hand) and T3SS expression (on the other). That we saw an increase in the expression of T3SS, T6SS, and exopolysaccharide biosynthetic genes suggests that signaling through the Rsm pathway may be dysregulated in EMC1. Second, and consistent with the proteomic observations, mexXY expression was up-regulated at a transcriptional level (↑≥2-fold). EMC1 exhibited down-regulation of pprA expression (↓2.4-fold). PprA and PprB are predicted to be part of a two-component regulatory system controlling membrane permeability. PprAB expression leads to increased membrane permeability and increased sensitivity to antibiotics, including aminoglycosides (46). The decreased expression of pprA may have been enough to prevent activation of the two-component system and to decrease cell permeability, thereby also contributing toward the elevated aminoglycoside resistance in EMC1. Another factor that impinges upon mexXY-oprM expression is the extracytoplasmic σ factor, SigX (47). sigX transcripts exhibited a slight decrease (↓1.3-fold) in expression in EMC1. This is potentially significant because SigX normally stimulates expression of the response regulator, PprB, and Gicquel et al. (47) have also reported elevated mexXY expression in a sigX mutant. Taken together, the decreased expression of both sigX and pprA, as well as the increased expression of the mexXY transcriptional activator, ArmZ (observed in the proteomics), likely explains the elevated gentamicin resistance of EMC1 (Table 1). The transcriptomic data also revealed that, in contrast with the observed up-regulation of mexXY, another RND-family efflux pump, mexGHI-opmD, was down-regulated in EMC1. This efflux system provides resistance to a variety of xenobiotics and has recently also been associated with binding a quorum-sensing molecule, the Pseudomonas quinolone signal (48). Although the precise physiological function(s) of MexGHI-OpmD remains to be elucidated, it has no known association with gentamicin resistance (49, 50). Finally, and consistent with the original aim of the study, cdrA transcription was down-regulated (↓1.4-fold) in EMC1.

Discussion

In this work, we serendipitously identified an SNP in the fusA1 gene of P. aeruginosa that gave rise to increased gentamicin resistance and global changes in expression of the T3 and T6 (HSI-I) secretion systems and exopolysaccharide biosynthetic pathways. FusA1 mutations are a common feature in certain clinical P. aeruginosa isolates (such as those derived from CF sputum) and are potentially a low-cost response to exposure to sublethal concentrations of aminoglycosides in vitro (20, 51, 52). However, to our knowledge, the wider phenotypic consequences of such mutations have not been explored further. The current work shows that at least one such fusA1 mutation can have selective, but potentially clinically significant, effects beyond conferring antibiotic resistance, by affecting the expression of key virulence factors. Early indications that the fusA1P443L mutant (EMC1) might be pleiotropically affected came from the unexpected observation that it had a larger growth defect (compared with the WT progenitor) in rich medium than it did in minimal medium. Our transcriptional data—showing that ribosomal gene expression is increased in EMC1—indicate that this is likely caused by the increased (but unmet, due to defective FusA1P443L function) translational demand during rapid growth in rich medium.

The elevated gentamicin resistance associated with EMC1 and other fusA1 mutants (20) appears to be driven by increased expression of the mexXY-encoded aminoglycoside efflux pump. This, in turn, is likely due to increased expression of the cognate transcriptional activator of mexXY expression, ArmZ. In addition, EMC1 also displayed transcriptional hallmarks indicative of decreased cell envelope permeability to aminoglycosides (linked to lower sigX and pprA/B expression). It seems highly unlikely that the fusA1 mutation itself contributes directly toward gentamicin resistance because “bypass” mutants in which gentamicin sensitivity was restored could be isolated. These appear to be due to the acquisition of second-site mutations because the fusA1P443L mutation was retained in all cases tested.

Interestingly, post-translational modifications (such as those carried out by ErmBP or TetO) that prevent the binding of
antibiotics to the ribosome or resistance-conferring mutations that block the binding of antibiotics to the ribosome also prevent induction of mexXY (53). Furthermore, although most of the antibiotics that are known to induce mexXY expression target the ribosome, not all of these are necessarily substrates of the pump (e.g., chloramphenicol). The mechanism underpinning this appears (at least in part) to be integrally linked with ArmZ expression. The armZ ORF is preceded by a short leader peptide, whose translation leads to the formation of a specific mRNA secondary structure. In this structure, a transcriptional terminator is exposed prior to the RNA polymerase reaching the armZ ORF, thereby preventing armZ (and thence mexXY) expression. However, when translation of the leader peptide is impaired, an alternative mRNA secondary structure forms in which the termination signal is occluded, allowing armZ expression (54). It requires no great leap of the imagination to infer that the impaired translation accompanying the fusA1P443L mutation could lead to a similar occlusion of the transcriptional terminator, enabling increased armZ expression (55). It is possible that a similar mechanism is responsible for some of the other pleiotropic (but nevertheless discrete) effects associated with the fusA1P443L mutation.

Residue Pro-443 (P. aeruginosa numbering) is conserved in >70% of FusA1 sequences across multiple phyla (55). The P443L substitution in the FusA1 of EMC1 introduces a hydrophobic residue (leucine) in place of a Pro-443 substitution in the FusA1 of EMC0 and plasmid construction. In this regard, we note that the correlation between the increased expression of the T3SS in EMC1 is not linked with mexXY expression per se. The elevated expression of T3 and T6 secretion systems in EMC1 was unexpected because these secretion systems may be co-regulated. For example, genes encoding the T3 and T6 systems have been reported to be up-regulated in a sigX mutant (47). In this regard, we note that sigX was slightly (1.3-fold) down-regulated in EMC1.

In summary, we have shown here that a mutation in the gene encoding a ribosome-recycling factor, FusA1, can lead to large-scale but discrete alterations in the physiology of P. aeruginosa. FusA1 mutants have been recently documented to confer resistance to aminoglycoside antibiotics (a phenotype that we confirm here), although their wider impact on virulence has not been reported. Our data show that one such fusA1 mutant displays greatly up-regulated expression of the T3SS machinery, as well as increased resistance to gentamicin. Taken together, our data indicate that these changes may be linked with sensing of diminished translational capacity in the fusA1 mutant, although additional work is required to confirm this and establish a mechanism. Consistent with this notion, it is worth recalling that treatment of P. aeruginosa with sub-MIC azithromycin (a macrolide targeting ribosome function) has also been shown to increase expression of the T3SS genes (59).

**Experimental procedures**

**Growth conditions**

Unless otherwise stated, P. aeruginosa PAO1 strains were grown at 37 °C in M9 minimal medium supplemented with 0.5% (w/v) glucose. Planktonic cultures were grown with vigorous aeration for the indicated time or to the indicated optical density. Late exponential cells were harvested at 7 h.

**EMC0 and plasmid construction**

EMC0 (the PcdrAB::lacZ reporter strain) was constructed by subcloning the PCR-amplified 320-bp upstream region of cdrAB in to mini-CTX-lacZ, immediately in front of the promoterless lacZ ORF. The reporter plasmid was introduced into PAO1 by electroporation, and transformants were selected for on 50 μg/ml tetracycline. Following integration of the construct into the chromosome, the mini-CTX backbone was
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removed by introducing pFLP2 into the transformants through biparental conjugation from β2163 (pFLP2). Transconjugants were selected for on 250 μg/ml carbenicillin. Transformants were streaked onto LB agar supplemented with 5% (w/v) sucrose to select for derivatives that had lost pFLP2 and then onto LB agar supplemented with 50 μg/ml tetracycline to verify successful excision of the tetracycline-resistance cassette. Loss of pFLP2 was confirmed through carbenicillin sensitivity. The resulting EMC0 was confirmed by PCR (and also by the whole-genome sequence data).

The complementation vectors, p fusA1 and p fusA1P443L, were constructed by PCR-amplifying the WT fusA1 and mutated fusA1P443L ORFs from PAO1- and EMC1-derived genomic DNA, respectively. The amplicons were then cloned into the PstI/HindIII sites in the MCS of pUCP20 (downstream of the lac promoter on the plasmid). The resulting plasmids were introduced into the recipient strains by electroporation and selection on 250 μg/ml carbenicillin. All plasmid constructs were confirmed by sequencing.

Plasposon mutagenesis

The pTnMod-OGm plasposon (22) was introduced into EMCO via triparental mating. EMCO was spotted onto solid agar with a helper Escherichia coli strain (HB101 (pRK21013)) and E. coli JM109 (pTnMod-OGm) for 18 h. The mixed colony was then resuspended, and transposon mutants were selected on agar media supplemented with 5 μg/ml gentalicin and 30 μg/ml X-Gal. After 30 h, gentalicin-resistant colonies were transferred to fresh gentamicin-supplemented plates and grown for a further 24 h. EMC1 was isolated as a pale blue colony at this stage.

β-gal activity

Aliquots (100 μl) of planktonic culture were harvested over a 10-h growth period and frozen at −80 °C in a 96-well microtiter plate. After collection was complete, the plate was defrosted at 37 °C, and 2 μl was transferred to a fresh 96-well microtiter plate. The samples were frozen at −80 °C for a second time, followed by thawing at room temperature. To quantitatively measure the level of β-gal production, 100 μl of PBS containing 20 mg/ml lysozyme and 250 μg/ml 4-methylumbelliferyl-β-D-galactoside was added to the cells. The reaction progress was monitored every 30 s for 30 min at 37 °C in a Gemini XPS fluorometer (Molecular Devices) using an excitation wavelength of 360 nm and emission wavelength of 450 nm.

Whole-genome sequencing

EMCO and EMC1 were sequenced using Illumina technology (HiSeq 2500 platform) by MicrobesNG (Birmingham, UK). Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina) following the manufacturer’s protocol with the following modifications: 2 ng of DNA were used instead of 1 ng, and PCR elongation time was increased to 1 min from 30 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid-handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit on a Roche light cycler 96 quantitative PCR machine. Libraries were sequenced on the Illumina HiSeq system using a 250-bp paired end protocol. Reads were adapter-trimmed using Trimomatic 0.30 with a sliding window quality cutoff of Q15. De novo assembly was performed on samples using SPAdes version 3.7, and contigs were annotated using Prokka 1.11. Genome alignment and analysis was carried out using Mauve Multiple Genome Alignment (60). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBankTM under accession numbers JAAGAW000000000 (EMCO) and JAAGAV000000000 (EMC1). The versions described in this paper are versions JAAGAW010000000 (EMCO) and JAAGAV010000000 (EMC1).

Motility assays

To detect twitching motility, 10 ml of 1.5% (w/v) agar-containing LB or M9 minimal medium/glucose was prepared in a 10-cm diameter Petri dish. Colonies were stabbed into the agar with a sterile toothpick, and the twitch halo was visualized after 24 h of incubation at 37 °C. To assess swimming motility, 25 ml of LB or M9 minimal media/glucose containing 0.3% (w/v) Bacto agar was dispensed into a 10-cm diameter Petri dish and allowed to solidify/surface-dry at room temperature for 30 min. Bacterial cultures were normalized to an OD600 of 1, and 3 μl of culture was dispensed as a stab column into the agar using a pipette. The halo of swimming bacteria was assessed after 8–18 h of incubation at 37 °C.

Exoenzyme secretion assays

Gelatinase activity was measured on solid medium plates containing 13 g/liter nutrient broth and 30 g/liter gelatin. Bacterial cultures were normalized to an OD600 of 1, and 5 μl was spotted onto the agar and left to soak in. Plates were incubated at 37 °C overnight. Gelatinase activity was visualized by flooding the plates with saturated ammonium sulfate solution for 15 min to reveal the proteolytic halo.

Exopolysaccharide secretion

Agar plates for the detection of exopolysaccharide production were prepared using 37 g/liter brain heart infusion broth supplemented with 50 g/liter sucrose and 0.8 g/liter Congo Red. Bacterial cultures were normalized to an OD600 of 1, and 10 μl was spotted onto the surface of the agar. The plates were incubated at 37 °C for 24 h. Exopolysaccharide production was determined semi-quantitatively through the amount of red pigmentation associated with the colony.

To quantitatively assess exopolysaccharide production, bacterial cultures were subcultured into fresh growth medium supplemented with 10 μg/ml Congo Red and incubated at 37 °C for 24 h on a rotating drum. The OD600 was measured, and the cells were pelleted at 3200 × g for 10 min at room temperature. This depletes any Congo Red bound to cell-associated polysaccharides; the greater the amount of polysaccharide sedimented, the lower the amount of Congo Red remaining in the culture supernatant. The optical density of the supernatant was measured at 495 nm. The A495 was then normalized against the original OD600 of the culture.
Siderophore detection

Bacterial cultures were harvested at late exponential phase. The cells were sedimented (3200 × g for 10 min), and the presence of siderophores in the cell-free culture supernatant was measured using the colorimetric SideroTech kit (Emergen Bio Inc.). A color change occurs as ferric iron in the kit reagent binds to siderophores present in the culture supernatants.

Western blotting

Following SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride membrane using a Trans-Blot Turbo Transfer System (Bio-Rad) with Trans-Blot Mini Transfer Packs. The membrane was incubated in blocking buffer (PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) semi-skimmed milk powder) overnight and then washed once in wash buffer (PBS and 0.1% (v/v) Tween 20). The membrane was then incubated for 1 h with a primary anti-PcrV antibody (1:5000) or anti-ICD antibody (1:10,000) or anti-Hcp1 antibody (1:10,000) (as indicated) in wash buffer. The membrane was then washed four times in wash buffer (5 min each wash), before the addition of IRDye 680RD (LI-COR) secondary antibody. This was incubated for 45 min. The membrane was washed four times, as before, and the protein bands were detected using an Odyssey CLx imaging system (LI-COR).

Proteomic analyses

Strains were grown with good aeration at 37 °C to late exponential phase (OD<sub>600</sub> of 0.6–0.8) in M9 minimal medium supplemented with 0.5% (w/v) glucose. To ensure retention of the complementation and empty vector plasmids, carbenicillin (250 μg/ml) was included in the starter culture, but not in the subculture used for proteomic analysis. We independently confirmed that the pUCP20 plasmid was retained by essentially all cells in the absence of selection over this sampling period (data not shown). For each strain, three biological replicates were analyzed. The proteome of a fourth EMCo replicate was analyzed by MS but was not included in this study. Cells from a 45-ml culture were harvested at 3200 × g for 30 min at 4 °C. The cell pellet was resuspended in PBS and sedimented a second time. Pellets were then resuspended in 800 μl of lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, pH 7.5) containing a Complete<sup>TM</sup> protease inhibitor mixture tablet (Roche Applied Science) and sonicated (3 × 5 s at 15 A, MSE microtip) on ice. Unlysed cells and debris were pelleted at 21,000 × g for 30 min at 4 °C. The protein concentration of the supernatant was determined using the DC protein assay (Bio-Rad). LC–MS/MS was performed by the Cambridge Centre for Proteomics. Protein samples were precipitated using ice-cold acetone. The samples were digested with trypsin with a specificity allowing for a maximum of one missed cleavage, carbamidomethylation of cysteine residues as a fixed modification, and oxidation of methionine and deamidation of glutamine/asparagine as variable modifications. Mass tolerance for precursor ions was 10 ppm, and for fragment ions, it was 0.8 Da. The dried peptides were reconstituted in 100 mM triethylammonium bicarbonate and labeled using 10-plex TMT (tandem mass tag) reagents according to the manufacturer’s protocol (Thermo Scientific). Tagged peptides were fractionated into 20 fractions by reverse-phase chromatography. Each fraction was run on 2-h gradients and was identified and quantified using a high-resolution Lumos Fusion Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-UPLC (Thermo Fisher Scientific). Synchronous precursor selection was used to ensure minimal TMT ratio compression (61). Proteome Discoverer (version 2.1.0.81; Thermo Fisher Scientific) was used to process raw MS data, with peptides identified using MASCOT (version 2.6.0, Matrix Science). Only high-confidence peptides defined by MASCOT with a 1% FDR were considered for peptide identification. FDRs were controlled with Percolator (version 2.05) at both the peptide-spectrum match and protein level using a strict threshold of 0.01 and a relaxed threshold of 0.05. Peptide and proteins were quantified by the Proteome Discoverer quantification module with the top three peptides used for area calculation. Proteomic data sets were analyzed with the empirical Bayes moderated t test implemented by the limma package (62). p values were corrected for multiple hypothesis testing using the Benjamini–Hochberg method (FDR ≤ 0.05). Differential expression was calculated based on normalized log<sub>2</sub> ratios. The MS/MS fragmentation data were searched against the National Center for Biotechnology Information (NCBI) Pseudomonas aeruginosa database (2017, 5584 entries) using the MASCOT search engine. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (63) partner repository with the data set identifier PXD017266.

Expression and purification of FusA1

For FusA1 purification, the WT and mutated fusA1 genes were PCR-amplified (from PAO1 and EMC1, respectively) and cloned into pET-19m. The resulting plasmids were introduced into Rosetta E. coli cells. The Rosetta cultures were grown in 1 liter of LB supplemented with 50 μg/ml carbenicillin and incubated at 37 °C to an OD<sub>600</sub> of 0.6–0.7. Isopropyl-β-D-thiogalactopyranoside was added to the cultures to a final concentration of 1 mM, and the culture was incubated at 20 °C for 24 h. Cells were centrifuged at 3430 × g, for 20 min at 4 °C. The cell pellet was resuspended in 10 ml of lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, pH 7.5) containing a Complete<sup>TM</sup> protease inhibitor mixture tablet (Roche Applied Science) and sonicated (3 × 5 s at 15 A, MSE microtip) on ice. Unlysed cells and debris were pelleted at 21,000 × g for 30 min at 4 °C. The protein concentration of the supernatant was determined using the DC protein assay (Bio-Rad). LC–MS/MS was performed by the Cambridge Centre for Proteomics. Protein samples were precipitated using ice-cold acetone. The samples were digested with trypsin with a specificity allowing for a maximum of one missed cleavage, carbamidomethylation of cysteine residues as a fixed modification, and oxidation of methionine and deamidation of glutamine/asparagine as variable modifications. Mass tolerance for precursor ions was 10 ppm, and for fragment ions, it was 0.8 Da. The dried peptides were reconstituted in 100 mM triethylammonium bicarbonate and labeled using 10-plex TMT (tandem mass tag) reagents according to the manufacturer’s protocol (Thermo Scientific). Tagged peptides were fractionated into 20 fractions by reverse-phase chromatography. Each fraction was run on 2-h gradients and was identified and quantified using a high-resolution Lumos Fusion Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-UPLC (Thermo Fisher Scientific). Synchronous precursor selection was used to ensure minimal TMT ratio compression (61). Proteome Discoverer (version 2.1.0.81; Thermo Fisher Scientific) was used to process raw MS data, with peptides identified using MASCOT (version 2.6.0, Matrix Science). Only high-confidence peptides defined by MASCOT with a 1% FDR were considered for peptide identification. FDRs were controlled with Percolator (version 2.05) at both the peptide-spectrum match and protein level using a strict threshold of 0.01 and a relaxed threshold of 0.05. Peptide and proteins were quantified by the Proteome Discoverer quantification module with the top three peptides used for area calculation. Proteomic data sets were analyzed with the empirical Bayes moderated t test implemented by the limma package (62). p values were corrected for multiple hypothesis testing using the Benjamini–Hochberg method (FDR ≤ 0.05). Differential expression was calculated based on normalized log<sub>2</sub> ratios. The MS/MS fragmentation data were searched against the National Center for Biotechnology Information (NCBI) Pseudomonas aeruginosa database (2017, 5584 entries) using the MASCOT search engine. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (63) partner repository with the data set identifier PXD017266.

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Intrinsic tryptophan fluorescence

The concentration of appropriately diluted purified His$_{6}$FusA1 was determined spectrophotometrically at 280 nm using a quartz cuvette (1-cm pathlength) and assuming MW = 78,738 and ε = 61,310 M$^{-1}$ cm$^{-1}$. EF-G protein was diluted in 2 ml of dialysis buffer to a final concentration of 0.8 μM. Intrinsic Trp fluorescence was measured in a thermostated (25 °C) quartz cuvette, on an FP-8300 spectrofluorometer (JASCO) using an excitation wavelength of 295 nm and an emission wavelength of 305–400 nm (0.5-nm intervals, 100 nm/min). All spectra were recorded in triplicate and then averaged.

Thermal shift analyses

Purified WT FusA1 or FusA1$^{P443L}$ (20-μl volume containing 20 μM protein) was mixed with SYPRO Orange dye and subjected to a thermal scan (25 °C to 95 °C) in a Roche Lightcycler 480, and the fluorescence was measured at 483 and 568 nm.

RNA extraction and sequencing

EMCO, EMC1 (both containing the pUCP20 empty vector), and EMC1$^*$ (containing pUCP20:fusA1) were cultured in M9 minimal medium supplemented with glucose, and samples were harvested at the late exponential phase of growth (OD$_{600}$ of 0.6–0.8) into RNAlater (Ambion). The samples were incubated at 4 °C for 15 min and pelleted at 21,000 × g for 20 min at 4 °C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and digested twice with on-the-column DNase I digestion, following the manufacturer’s guidelines. The concentration and purity of the resulting RNA were measured using a NanoDrop ND-1000 spectrophotometer. The absence of contaminating proteins and organic compounds was indicated by an A$_{260/280}$ ratio of 1.8–2.0 and A$_{260/230}$ nm of 2.0–2.2. Total RNA was sent to GATC Biotech (Konstanz, Germany) for rRNA depletion and RNA sequencing using the Illumina platform (10 million reads per sample, single read, 1 × 50 bp). The RNA-Seq reads were processed using Fastqc and were mapped to the PA01 genome and analyzed using the Tuxedo Suite package. The principal component analysis plot and the volcano plots were constructed in R. RNA-Seq data have been deposited in the ArrayExpress database at EMBL-EBI (64) under accession number E-MTAB-8690.

Data availability

The data supporting the findings of this study are available within this article (and the supporting material) or can be accessed through publicly available repositories. Whole-genome sequencing data sets generated in this study are openly available in GenBank™, DDBJ, and ENA, under the accession numbers JAAGAW000000000 (EMC0) and JAAGAV000000000 (EMC1). The versions described in this paper are versions JAAGAW010000000 (EMC0) and JAAGAV010000000 (EMC1). Proteomics data generated in the study are openly available via the ProteomeXchange Consortium using the identifier PXD017266. RNA-Seq data sets generated in this study are openly available in the ArrayExpress database under the accession number E-MTAB-8690.

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Abbreviations—The abbreviations used are: CF, cystic fibrosis; X-Gal, 5-bromo-4-chloro-3-indolyl β-d-galactoside; T3, Type III; T3SS, Type III secretion system; T6, Type VI; T6SS, Type VI secretion system; EF-G, elongation factor G; LB, lysogeny broth; WGS, whole-genome sequencing; MIC, minimum inhibitory concentration; FC, fold change; FDR, false discovery rate; OD, optical density.

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