LETTERS

Aminoglycoside antibiotics induce bacterial biofilm formation

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Biofilms are adherent aggregates of bacterial cells that form on biotic and abiotic surfaces, including human tissues. Biofilms resist antibiotic treatment and contribute to bacterial persistence in chronic infections[1,2]. Hence, the elucidation of the mechanisms by which biofilms are formed may assist in the treatment of chronic infections, such as *Pseudomonas aeruginosa* in the airways of patients with cystic fibrosis[2]. Here we show that subinhibitory concentrations of aminoglycoside antibiotics induce biofilm formation in *P. aeruginosa* and *Escherichia coli*. In *P. aeruginosa*, a gene, which we designated aminoglycoside response regulator (*arr*), was essential for this induction and contributed to biofilm-specific aminoglycoside resistance. The *arr* gene is predicted to encode an inner-membrane phosphodiesterase whose substrate is cyclic di-guanosine monophosphate (c-di-GMP)—a bacterial second messenger that regulates cell surface adhesiveness[3]. We found that membranes from *arr* mutants had diminished c-di-GMP phosphodiesterase activity, and *P. aeruginosa* cells with a mutation changing a predicted catalytic residue of Arr were defective in their biofilm response to tobramycin. Furthermore, tobramycin-inducible biofilm formation was inhibited by exogenous GTP, which is known to inhibit c-di-GMP phosphodiesterase activity[4]. Our results demonstrate that biofilm formation can be a specific, defensive reaction to the presence of antibiotics, and indicate that the molecular basis of this response includes alterations in the level of c-di-GMP.

Most antibiotics of clinical relevance are derivatives of naturally occurring microbial products that probably function in microbial competition within environmental niches[5]. The aminoglycosides are a class of clinically important antibiotics that have been widely used to treat chronic bacterial infections of the heart, lung and urinary tract[6]. Tobramycin, an aminoglycoside produced by the bacterium *Streptomyces tenebrarius*, is commonly used because of its enhanced effectiveness against infections with the opportunistic pathogen *P. aeruginosa* (ref. 6). Nevertheless, clinical isolates of *P. aeruginosa* possess an inducible resistance to tobramycin[7], suggesting that *P. aeruginosa* responds adaptively to aminoglycoside exposure. Because both *P. aeruginosa* and *S. tenebrarius* are present in soil[8], we proposed that *P. aeruginosa* had evolved adaptive responses to tobramycin before the clinical use of antibiotics, and that one such response was the formation of antibiotic-resistant biofilms. This response would be advantageous for *P. aeruginosa* both in the soil, when encountering an aminoglycoside-producing bacterium such as *S. tenebrarius*, and in human hosts receiving antibiotic therapy, where bacteria may encounter variable antibiotic concentrations[9]. Such situations include the chronic airway infections of cystic fibrosis (CF) patients, in which *P. aeruginosa* frequently reaches densities of 10^9 viable cells per ml of sputum[10]. Upon treatment with tobramycin in aerosol form, a fraction of these bacteria may inevitably be exposed to subinhibitory levels of antibiotic. Indeed, tobramycin rarely eradicates these infections, even before the appearance of tobramycin-resistant mutants[10].

To study the earliest physiological response of *P. aeruginosa* to tobramycin, we used concentrations ≤0.3 times the minimal inhibitory concentration (MIC) of 1 μg ml^{-1}. These tobramycin concentrations did not measurably alter the growth rate of *P. aeruginosa* strain PAO1 in Mueller-Hinton broth (MHB; Fig. 1a and Supplementary Information); nor did they alter global protein synthesis as determined by measurement of total cellular protein in the PAO1 strain and β-galactosidase activity in strains with *lacZ* translational fusions to several genes (*hcnB*, *phzD*, *pqsH*, *katB*; data not shown). Despite this lack of inhibition, a range of subinhibitory tobramycin concentrations induced biofilm formation in PAO1 (Fig. 1b, c). A peak induction in biofilm mass of 3.4-fold occurred during growth in

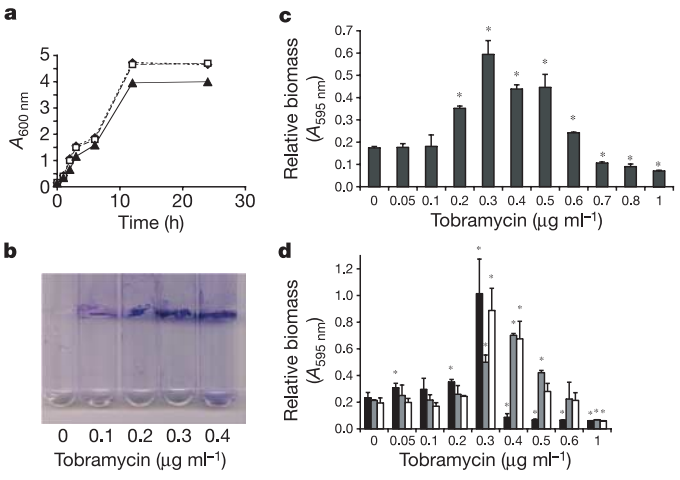

Figure 1 | Phenotypic effects of tobramycin on *P. aeruginosa* and *E. coli*. **a**, Growth curve of shaken cultures of *P. aeruginosa* strain PAO1 in 0 (filled diamonds), 0.3 (open squares) and 0.4 (filled triangles) μg ml^{-1} tobramycin (MIC 1 μg ml^{-1}). **b**, Tobramycin-induced biofilm formation in PAO1 cultured on glass culture tubes (after 24 h static growth) visualized by crystal-violet staining. **c**, Tobramycin-induced biofilm formation in PAO1 cultured on plastic microtitre plates. Note that biofilm formation in the absence of the drug is lower on glass (panel **b**) than in polystyrene plates. Results are averages of 8 replicates ±s.e.m. and are representative of 10 independent experiments. Asterisk, $P < 0.001$ as compared with PAO1 exposed to no drug. **d**, Tobramycin-induced biofilm formation in *E. coli* cultured on plastic microtitre plates. Three strains were tested, with the results for individual strains indicated by black, grey or white bars, respectively. Results are averages of 8 replicates ±s.e.m. Asterisk, $P < 0.001$ as compared with the same strain exposed to no drug.

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the presence of tobramycin at 0.3 times the MIC (Fig. 1c)—an increase in biomass similar to that associated with *P. aeruginosa* mutants with increased propensities for biofilm formation[2,11]. Tobramycin induced biofilm formation in three different growth media (M63 salts supplemented with glucose and casamino acids, MHB, Luria broth), on several abiotic surfaces (borosilicate glass, polystyrene, polypropylene, polycarbonate) and in 13 out of 14 *P. aeruginosa* clinical isolates tested (Fig. 1b, c and Supplementary Information and data not shown).

For both Gram-negative and Gram-positive bacteria, subinhibitory antibiotic treatment can stimulate production of exopolysaccharides[12,13]. Subinhibitory levels of the β-lactam antibiotic imipenem augment production of the *P. aeruginosa* exopolysaccharide alginate, and leads to increased biofilm volume[13]. However, *P. aeruginosa algD* mutants (which are defective in alginate production) and the parental strain PAO1 demonstrated equivalent tobramycin-induced biofilm formation (data not shown). Furthermore, the tobramycin-induced increase in biomass (Fig. 1c) corresponded to an increase in biofilm colony-forming units (CFU) (Supplementary Information), indicating that it resulted primarily from an increase in cell number and not an increase in extracellular matrix.

We assayed other antibiotics at a range of concentrations (relative to the MIC) equivalent to those used for tobramycin. Three additional aminoglycoside antibiotics induced biofilm formation, although tobramycin had the strongest effect. The maximum induction by amikacin, streptomycin and gentamicin was 75%, 66% and 25% of that by tobramycin, respectively. In contrast, polymyxin B (a peptide antibiotic that is cationic like aminoglycosides and interacts with membranes) had no effect on biofilm formation; nor did the protein synthesis inhibitor chloramphenicol or the cell wall synthesis inhibitor carbenicillin (data not shown). Therefore, biofilm induction by tobramycin is unlikely to be solely due to non-specific protein synthesis inhibition, cell damage or interaction with cell membranes through positive charge; rather, it appears to be a specific response by *P. aeruginosa* to aminoglycosides.

In order to test whether this response was present in other Gram-negative bacteria, we examined the enteric bacterium *E. coli*. Subinhibitory levels of tobramycin induced biofilm formation in three clinical isolates of *E. coli* from three patients with bacteraemia (Fig. 1d). As with *P. aeruginosa*, this induction occurred in a range of tobramycin concentrations centred on 0.3 $\mu g\,ml^{-1}$ (0.3 × the MIC for *E. coli*). Such conservation suggests that tobramycin activates a signalling pathway present in both *P. aeruginosa* and *E. coli*.

Signalling by the second messenger c-di-GMP is a good candidate for such a conserved system as this dinucleotide regulates cell adhesiveness in a diverse range of bacteria[3,14,15]. The PAO1 genome includes at least 38 genes that are predicted to encode a regulator of intracellular c-di-GMP levels. These proteins contain either a GGDEF domain (found in putative cyclases for c-di-GMP synthesis), an EAL domain (found in putative phosphodiesterases for c-di-GMP degradation), or both[16]. To identify which, if any, of these genes might be involved in signalling the presence of tobramycin, we screened the relevant transposon-insertion mutants of PAO1 (ref. 17) for tobramycin-inducible biofilm formation. We reasoned that the inactivation of a gene involved in signalling the presence of tobramycin would result in a strain that showed reduced biofilm induction by tobramycin, but normal biofilm production in the absence of tobramycin, relative to wild-type cells. Many of the mutants had altered biofilm formation even in the absence of tobramycin (Supplementary Information), and these were not analysed further.

Three strains, each with a different insertion mutation in the monocistronic open reading frame PA2818, were defective for tobramycin-induced biofilm formation (Fig. 2a, b and Supplementary Information). We designated this gene *arr*, for aminoglycoside response regulator. The *arr* gene is predicted to encode an inner-membrane protein with two transmembrane domains, a periplasmic domain that could transduce an environmental stimulus, and an EAL domain (Fig. 3a). Database searches revealed a similar domain architecture in the gene products of many Gram-negative bacteria, including *E. coli*. Complementation with a plasmid expressing *arr* restored wild-type tobramycin-induced biofilm formation in an *arr* mutant (Fig. 2a), indicating that Arr is necessary for this response. There was no complementation with a plasmid expressing a mutant *arr* gene encoding a protein in which the conserved glutamate residue of the EAL domain was replaced with an alanine (E297A) (Fig. 2d). As this mutation has abolished the biological activity attributed to other EAL domain proteins[18], this result suggested that Arr phosphodiesterase activity is required for tobramycin-induced biofilm formation.

Consistent with Arr having c-di-GMP phosphodiesterase activity, membranes from *arr* mutant cells were 54% less active in degrading c-di-GMP than membranes from PAO1 cells (Fig. 3b); this is a surprising finding given that 23 of the 38 predicted PAO1 c-di-GMP regulators are expected to be transmembrane proteins. Wild-type phosphodiesterase activity was restored to the *arr* mutant by a plasmid expressing *arr* (Fig. 3b), but not by a plasmid expressing the gene encoding the E297A Arr mutant (data not shown). Furthermore, tobramycin-inducible biofilm formation in wild-type cells was inhibited by exogenous GTP—a c-di-GMP phosphodiesterase inhibitor[4] (Supplementary Information). Based on these results, we propose that tobramycin, either directly or indirectly, enhances the phosphodiesterase activity of the Arr cytoplasmic EAL domain, leading to c-di-GMP inactivation and augmented biofilm formation (Fig. 3a).

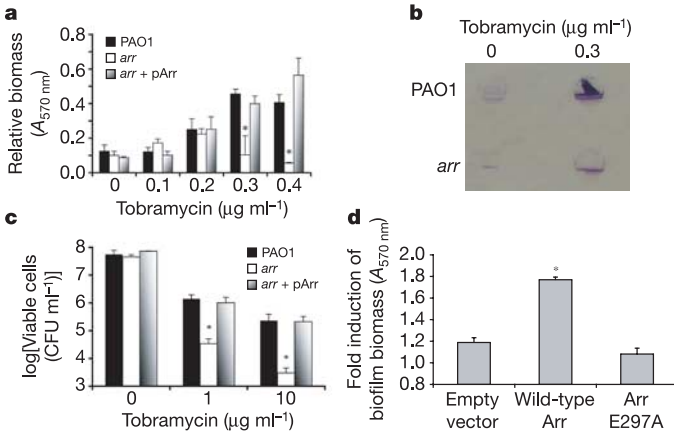

Figure 2 | The role of *P. aeruginosa arr* in tobramycin induction of biofilm formation and biofilm antibiotic resistance. **a**, Microtitre plate biofilm assay with the parental PAO1 strain, the *arr* mutant strain, and the *arr* mutant strain carrying a plasmid expressing the wild-type *arr* gene (pArr). Results are averages of 8 replicates ±s.e.m. and are representative of three independent experiments. Asterisk, $P < 0.001$ as compared with PAO1 cultured in the same tobramycin concentration. **b**, Crystal-violet-stained biofilms of PAO1 and the *arr* mutant strain grown for 24 h on glass coverslips spanning the air–liquid interface of standing cultures. The dark portion above the air–liquid interface biofilm in tobramycin-induced PAO1 is adherent pellicle. **c**, Microtitre plate biofilm antibiotic resistance assay in which biofilms of the indicated strains were challenged with tobramycin at the concentrations shown, and the survival of suspended biofilm cells determined. Results are the average of three experiments ±s.e.m. Asterisk, $P < 0.001$ as compared with PAO1 biofilms exposed to the same drug concentration. **d**, The indicated plasmids were introduced into the *arr* mutant strain. Fold induction of biofilm formation by 0.3 $\mu g\,ml^{-1}$ tobramycin for each transformant was determined (average ±s.e.m. of 6 replicates). Asterisk, $P < 0.001$ as compared with cells carrying the empty vector. Cells were grown in 500 $\mu g\,ml^{-1}$ carbenicillin for plasmid maintenance.

Although Arr promotes biofilm formation, expression of EAL-type regulators in other organisms has been linked to a reduction in biofilm formation[15]; this is in apparent contradiction with our model (Fig. 3a). However, inactivation of putative c-di-GMP regulatory proteins (with either GGDEF, EAL, or both domains) had remarkably varied effects on basal levels of biofilm formation in PAO1 (Supplementary Information). This indicates a complex relationship between biofilm formation and the expression of individual regulators, which could involve the localized regulation of discrete cytoplasmic c-di-GMP pools. The *Caulobacter crescentus* GGDEF-type regulator PleD (ref. 14) and the *P. aeruginosa* GGDEF/EAL-type regulator FimX are both localized to one cell pole[3,14,19]. Similarly, the Arr periplasmic domain could mediate the formation of localized protein complexes.

Multiple cell surface appendages mediate bacterial aggregation and facilitate biofilm formation. Flagella and pili are involved in biofilm formation[11,20] and are regulatory targets of c-di-GMP (refs 14, 19). It was possible that tobramycin and Arr were regulating biofilm formation by altering these surface appendages. Indeed, subinhibitory tobramycin concentrations inhibited PAO1 flagellar motility in a dose-dependent manner, and inhibition was reduced in an *arr* mutant (Supplementary Information). Nevertheless, tobramycin did not affect type IV pili-mediated twitching motility, and PAO1 *fliC* mutants (lacking flagella) and *pilA* mutants (lacking type IV pili) showed tobramycin-induced biofilm formation that was equivalent to wild-type levels (data not shown). The *arr* mutant was also motile in assays for twitching (data not shown) and flagellar swimming (Supplementary Information), and polymyxin B inhibited flagellar swimming (Supplementary Information) without any effect on biofilm formation (data not shown). Therefore, it is unlikely that tobramycin or Arr affect biofilm formation simply via these surface appendages.

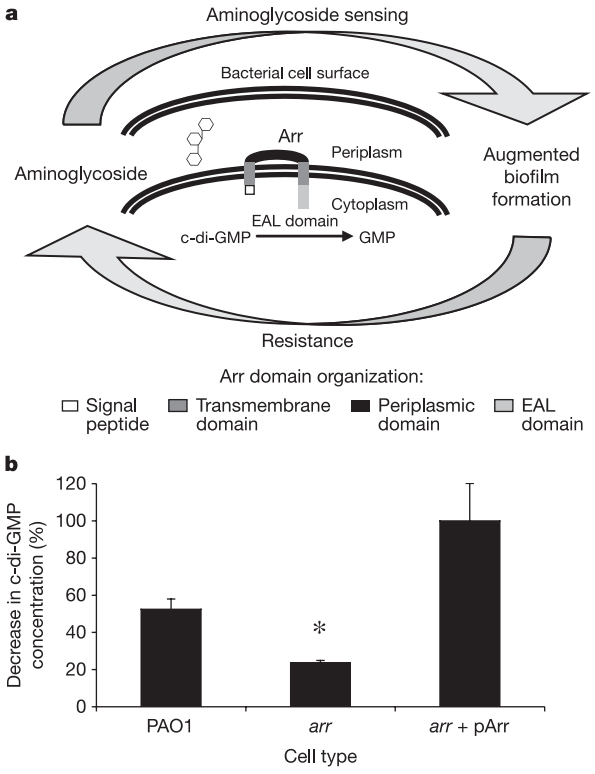

Figure 3 | The role of c-di-GMP in the biofilm response to aminoglycosides. a, A model for aminoglycoside effects on *P. aeruginosa*. Aminoglycoside antibiotics such as tobramycin (indicated by the three-ringed structure) could act as first messengers that trigger changes—mediated either by binding directly to proteins such as Arr or indirectly through intermediary molecules—in the level of the second messenger c-di-GMP. The proposed domain organization of Arr is indicated, including the location of the carboxy-terminal EAL domain. **b**, Effect on c-di-GMP concentration of washed cell membranes containing 0.4 mg of protein from either the PAO1 strain, the *arr* mutant strain, or the *arr* mutant strain with a plasmid expressing the wild-type *arr* gene after incubation with 10 μM c-di-GMP. Results are averages of at least 3 replicates ±s.d., and are representative of four separate experiments. Asterisk, $P < 0.001$ as compared with PAO1.

Because biofilms are associated with increased antibiotic resistance[1,2], we tested whether Arr had a role in biofilm-mediated antibiotic resistance as well as biofilm formation. In a peg biofilm assay that was recently proposed for clinical use[21], biofilms of *arr*-mutant cells were approximately 100-fold more susceptible to tobramycin killing than PAO1 biofilms, and the wild-type phenotype was restored by expressing *arr* from a plasmid (Fig. 2c). Decreased biofilm tobramycin-resistance of the *arr* mutant was also observed in a standard biofilm crystal violet staining assay (data not shown), and by measuring CFU in colony biofilms (Supplementary Information). In contrast, planktonic cultures of *arr*-mutant cells exhibited the same killing by tobramycin as cultures of wild-type cells (MIC of 1 μg ml^{-1}). This indicates that *arr*, like *ndvB* (ref. 1), is a genetic determinant of biofilm-mediated antibiotic resistance in *P. aeruginosa*. The contribution of Arr to biofilm tobramycin-resistance could be clinically relevant, as improvement of lung function in CF patients treated with tobramycin in aerosol form correlates with an approximate 100-fold reduction in *P. aeruginosa* CFU (ref. 10); this is equivalent to the difference in killing between wild-type and *arr*-mutant biofilms treated with tobramycin (Fig. 2c).

Another *P. aeruginosa* EAL-type regulator, PvrR, plays a role in the formation of small colony variants (SCVs) that are hyperadherent and antibiotic resistant[22]. Mutation of *pvrR* increased the frequency of appearance of SCVs growing in the presence of high concentrations of the aminoglycoside kanamycin[22]. This raised the possibility that mutation of *arr* altered the frequency of such variants with a consequent change in tobramycin-induced biofilm formation. To test this, we isolated and examined resuspended cells from PAO1 and *arr*-mutant biofilms grown with and without subinhibitory tobramycin. The frequency of SCVs was equivalent in each of the four cell populations (data not shown). In addition, we tested these resuspended cells for their ability to form new biofilms in the absence of antibiotic, and for their tobramycin susceptibilities in both static and shaken planktonic cultures. None of these characteristics were altered, either by mutation of *arr* or by subinhibitory tobramycin treatment (Supplementary Information). Therefore, subinhibitory tobramycin does not enrich for relatively resistant or adherent genetic variants, and the *arr* mutant phenotype is not due to the altered frequency of such variants.

Taken together, our results suggest that inhibiting the activity of EAL-type regulators such as Arr might be of therapeutic benefit early in *P. aeruginosa* chronic infections: particularly airway infections in which the aerosol form of tobramycin is widely used. It might also be beneficial in acute disease, as the mutation of *pvrR* decreased *P. aeruginosa* virulence in burned mice[23]. However, clinical strain variability could complicate such therapeutic manipulation of c-di-GMP metabolism. The *pvrR* locus of strain PA14 is contained on a genetic island that is absent in strain PAO1 (ref. 23). Similarly, a genomic microarray analysis suggested that *arr* was absent or divergent in some *P. aeruginosa* isolates[24]. This variability notwithstanding, 13 of the 14 CF isolates we tested demonstrated tobramycin-induced biofilm formation, while the one remaining isolate appeared to lack *arr* by polymerase chain reaction (PCR) analysis (data not shown). Therefore, the relevant characteristic conserved among these strains and with *E. coli* (Fig. 1c, d) is antibiotic induction of biofilm formation, probably through c-di-GMP as a second messenger. This biofilm response could contribute to differences in therapeutic outcome upon antibiotic treatment, such as in CF where not all patients respond positively to tobramycin aerosols[10], and the ability to manipulate c-di-GMP metabolism may have

therapeutic value by increasing bacterial susceptibility to standard therapy.

There is growing evidence that bacteria respond specifically and defensively to subinhibitory antibiotic concentrations[5,25]. The evidence presented here indicates that Gram-negative bacteria can respond to aminoglycosides by forming antibiotic-resistant biofilms—perhaps only one of many strategies used to counter antibiotic production by Gram-positive soil bacteria such as the Streptomycetes. In *P. aeruginosa*, this biofilm response requires Arr—a regulator that alters c-di-GMP levels (Fig. 3a). Both in soil and within animals, c-di-GMP regulation could contribute to the diversity of possible outcomes for bacterial communities challenged with antibiotics.

METHODS

Bacterial strains, chemicals and media. The PAO1 strain was obtained from B. Iglewski through C. Manoil, and the transposon-insertion mutants from M. Jacobs[17]. *E. coli* isolates were from patients with bacteraemia at the Massachusetts General Hospital (archived by S. Miller), and *P. aeruginosa* longitudinal clinical isolates were from young CF patients less than 8 yr old (provided by J. Burns[26]). PAO1 mutants containing a transposon TN*5*-derived insertion element[17] in the gene PA2818 that were analysed in detail included the 50022 (PA2818::IS*phoA*/hah-Tc), 17026 (PA2818::IS*lacZ*/hah-Tc) and 5339 (PA2818::IS*lacZ*/hah-Tc) mutants; these mutants are described more fully at http://www.genome.washington.edu/UWGC/pseudomonas/index.cfm. Mutants in other genes are described in the Supplementary Methods. All data shown for mutants in the PA2818 gene are for PAO1 mutant 50022; results were confirmed with PAO1 mutants 17026 and 5339. Bacteria were grown at 37 °C in MHB (Difco) unless otherwise indicated. Polymyxin B was purchased from Amersham Life Sciences. GTP, as well as tobramycin sulphate and all other antibiotics, were obtained from Sigma. Unlabelled and ^{18}O-labelled c-di-GMP was synthesized, purified and characterized as described[27].

Phenotypic assays. *P. aeruginosa* PAO1 was inoculated in duplicate onto Mueller-Hinton agar (MHA) with 0.3% agar (to characterize swimming motility) or beneath MHA with 1.5% agar (to characterize twitching motility), each with and without antibiotics, and then incubated at 37 °C for 12 h, essentially as described[20]. For use as an inoculum for MIC determination (by broth microdilution), killing assays, growth curves and biofilm assays, overnight cultures of bacteria grown in MHB were diluted approximately 1:100 with MHB (to a density of 10^7 CFU ml^{-1}). Antibiotics were added to this inoculum as indicated. Cell number was determined by measuring CFU (by plating serial dilutions of cultures onto LB agar) or by measuring the absorbance at 600 nm ($A_{600\,nm}$) of suspended cells. Biofilm formation was routinely quantified by measuring either $A_{570\,nm}$ or $A_{595\,nm}$ (which gave equivalent results) of crystal-violet staining of adherent cells as described previously[21]. To assay the induction of biofilm formation, biofilms were grown for 24 h without shaking at 37 °C either in glass culture tubes, on glass cover slips, or in wells of untreated 96-well polystyrene microtitre plates (Nunc) using 100 μl of culture per well (8 duplicates per condition per experiment). To assay biofilm antibiotic resistance and to confirm biofilm induction, biofilms were grown in microtitre plates without antibiotics as above and with a lid containing 96 polystyrene pegs (Nunc) such that a peg was inserted into each well. The pegs with adherent biofilms were removed after 24 h, washed three times in water, and placed into a new microtitre plate with fresh MHB supplemented with antibiotics. After static incubation for 24 h at 37 °C, the pegs were removed, washed three times with water, inserted into a 96-well plate containing fresh MHB, centrifuged at 1,811 *g* for 20 min, and then sonicated at approximately 25 °C for 5 min (using a Branson 1510 water bath; Branson) to remove and disperse adherent cells for CFU determination as described[21]. Consistent removal of the biofilm was confirmed by crystal-violet staining of the pegs. For microtitre biofilm assays, reported values are the mean of at least three replicates (with the error calculated as s.e.m.). Student's two-tailed *t*-test was used to establish the significance of differences between two means. In parallel with each biofilm experiment, an MIC was determined for the planktonic cells in the culture used as the inoculum. For all of the *P. aeruginosa* strains derived from PAO1, and for the *E. coli* strains tested, the MIC of tobramycin was consistently 1 μg ml^{-1}, including for resuspended biofilm-grown *P. aeruginosa*.

Cloning of *arr* and complementation experiments. A PCR fragment containing the gene PA2818 (nucleotides 3,170,500–3,174,000 in the *P. aeruginosa* PAO1 genome) was cloned into the *Eco*RI and *Hind*III sites of pUCP18. The resulting plasmid was introduced into PAO1 and the PA2818 (*arr*) 50022 mutant by electroporation and selection for carbenicillin resistance using standard methods. These transformants were found to retain the plasmid for over 36 h of growth in the absence of selection. Therefore, biofilm experiments with the strain carrying the resulting plasmid with the wild-type *arr* gene were performed in the absence of carbenicillin, except as indicated.

Measurement of c-di-GMP. Washed cell membranes were prepared and assayed for c-di-GMP degrading activity essentially as described[28]. Overnight cultures of *arr* mutant and wild-type PAO1 were diluted 1:100 in MHB and grown statically at 37 °C for 18 h. The cells were collected by centrifugation, the pellet resuspended in TME buffer (50 mM Tris-HCl pH 8, 0.9 mM EDTA, 10 mM $MgCl_2$) and the cells disrupted by sonication on ice. Unbroken cells were removed by centrifuging at 3,000 r.p.m. for 5 min. Membranes were separated from the supernatant by centrifuging at 14,000 r.p.m. for 30 min. The resulting pellets of washed membranes were resuspended in TME buffer, and protein concentration determined by a modified Lowry assay (BioRad). Aliquots of membrane corresponding to the indicated amount of protein were resuspended in 200 μl TME buffer with 10 μM c-di-GMP. The mixture was incubated for 10 min at 30 °C, and the reaction halted by boiling at 100 °C for 2 min. Membranes were removed by centrifugation at 14,000 r.p.m. for 30 min. The supernatant was analysed for c-di-GMP content as described in the Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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