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Cyclic-di-GMP levels affect *Pseudomonas aeruginosa* fitness in the presence of imipenem

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Summary

A large number of genes coding for enzymes predicted to synthesize and degrade 3′–5′-cyclic diguanylic acid (c-di-GMP) is found in most bacterial genomes and this dinucleotide emerged as an intracellular signal-controlling bacterial behaviour. An association between high levels of c-di-GMP and antibiotic resistance may be expected because c-di-GMP regulates biofilm formation and this mode of growth leads to enhanced antibiotic resistance. However, a clear understanding of this correlation has not been established. We found that increased levels of c-di-GMP in *Pseudomonas aeruginosa* improve fitness in the presence of imipenem, even when grown as planktonic cells. *P. aeruginosa* post-transcriptionally regulates the amounts of five porins in response to c-di-GMP, including OprD, responsible for imipenem uptake. Cells with low c-di-GMP levels are consequently more sensitive to this antibiotic. Main efflux pumps or β-lactamase genes did not show altered mRNA levels in *P. aeruginosa* strains with modified different c-di-GMP concentrations. Together, our findings show that c-di-GMP levels modulate fitness of planktonic cultures in the presence of imipenem.

Introduction

Pseudomonas aeruginosa is a versatile gamma-proteobacterium that behaves as an opportunistic pathogen over a broad range of hosts (Lyczak *et al*., 2000). The ability of *P. aeruginosa* to form biofilms contributes to its virulence and adaptation to diverse environments (Costerton *et al*., 1995; 1999; Govan and Deretic, 1996). The biofilm lifestyle requires reprogramming of the expression profile of planktonic cells. c-di-GMP (3′-5′-cyclic diguanylic acid) is a second messenger that plays an important role in this regulation. High c-di-GMP levels are linked to biofilm formation and low levels with a motile planktonic lifestyle (Romling *et al*., 2013). This cyclic dinucleotide controls gene expression (Hengge, 2009; 2010), acting at the level of transcription by affecting the activity of regulatory proteins or post-transcriptionally, binding to riboswitches. c-di-GMP can also protect bacterial surface-exposed proteins against proteolysis and it is an allosteric effector for some enzymes (Hengge, 2010; Boyd and O'Toole, 2012). c-di-GMP is synthesized from GTP di-guanylate cyclases that bear a conserved GGDEF domain, and it is hydrolysed by phosphodiesterases with EAL or HD-GYP domains, which cleave c-di-GMP to pGpG or GMP respectively (Romling *et al*., 2013). The genome of *P. aeruginosa* strain PA14 presents 40 genes coding for proteins associated with c-di-GMP metabolism, highlighting a broad regulatory role of the di-nucleotide in this species (Lee *et al*., 2006).

Samples of *P. aeruginosa* isolated from the lungs of cystic fibrosis patients often contain small colony variants (SCV) with high intracellular levels of c-di-GMP, characterized by an aggregative phenotype associated with persistence, antibiotic resistance, adhesion and exopolysaccharide production (Jimenez *et al*., 2012).

Pseudomonas aeruginosa displays high intrinsic resistance to antibiotics, which mainly results from the restricted passage of these drugs through the outer membrane and energy-dependent efflux. Imipenem is a β-lactam antibiotic broadly used against *P. aeruginosa* and has been associated with the emergence of resistance to other antibiotics of several classes, such as ciprofloxacin, ceftazidime and piperacillin/tazobactamin (Carmeli *et al*., 1999; Miliani *et al*., 2011).

High c-di-GMP leads to a biofilm growth mode that has been proven to enhance antibiotic resistance (Mah and O'Toole, 2001; Drenkard, 2003; Harmsen *et al*., 2010; Hoiby *et al*., 2010). However, there is no evidence that c-di-GMP has a direct role in adapting cells to the presence of antibiotics. One report shows that *P. aeruginosa* cells with low c-di-GMP levels are more resistant to the

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antimicrobial peptide colistin than cells with high c-di-GMP levels (Chua *et al*., 2013). In the phytopathogenic bacterium *Xylella fastidiosa*, subinhibitory concentrations of tobramycin increased the expression of the *eal* gene coding for a phosphodiesterase, and an *eal* mutant was more susceptible to this antibiotic (de Souza *et al*., 2013). In this work, we investigated whether there is a correlation between c-di-GMP levels in planktonic cultures and adaptation in the presence of an antibiotic. We chose imipenem as a starting point because of its clinical relevance (Lautenbach *et al*., 2010).

Results

The *P. aeruginosa* PA14 genome encodes 40 different proteins with GGDEF, EAL and/or HD-GYP domains (Kulasakara *et al*., 2006; Lee *et al*., 2006). Uncovering the role of c-di-GMP with single mutant strains is precluded by the large number of genes involved in c-di-GMP metabolism because the absence of one enzyme may lead to compensatory effects, causing alterations in the expression and/or activity of the remaining ones. To overcome this limitation in studying mutants, we used strains overexpressing either a phosphodiesterase or a diguanylate cyclase with established enzymatic activity (Kulasakara *et al*., 2006) to reveal processes regulated by c-di-GMP levels. Strains RB210 and RB211 were constructed by overexpression of the EAL phosphodiesterase PvrR or the GGDEF diguanylate cyclase PA14_72420 (PA5487 in the PAO1 genome), respectively, under the control of an arabinose-inducible promoter integrated in the *ctx* site of PA14 chromosome (Table 1).

Measurements of intracellular c-di-GMP in RB210 and RB211 cells by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) showed a sixfold difference in c-di-GMP concentration between the strains under induced conditions (Fig. 1). These strains also presented altered biofilm and motility phenotypes (Supporting Information Fig. S1), which correlate well with high or low c-di-GMP concentration. These results confirm that overexpression of the c-di-GMP metabolic enzymes PvrR and PA14_72420 alters the behaviour of *P. aeruginosa*.

High c-di-GMP levels improve growth in the presence of subinhibitory imipenem concentration

The connection between high c-di-GMP levels, increased biofilm formation and antibiotic resistance seems straightforward. However, the exact role of c-di-GMP in antibiotic resistance, uncoupled from its role in biofilm formation, is unknown. To test if c-di-GMP is involved in this process, the minimum inhibitory concentration (MIC) for imipenem was determined in planktonically grown cultures of strains with c-di-GMP levels higher or lower than wild type. All strains presented the same MIC as PA14 (1 μg ml^{-1}), but it was evident that the strain with higher c-di-GMP levels grew at a faster rate when exposed to sub-MIC imipenem concentrations (Supporting Information Fig. S2). Therefore, to uncover small differences in the resistance threshold, growth of those strains was followed over time. Strain RB211 (high c-di-GMP levels) presented growth improvement when compared with wild-type PA14 in 0.5 μg ml^{-1} imipenem, whereas no differences were observed without antibiotic or at MIC (Fig. 2). These results were consistent when cultures were grown in 48- (Fig. 2) and 96-well plates in a Spectramax Paradigm instrument (Molecular Devices, Sunnyvale, CA, USA), in 100-well plates in a Bioscreen (Growth Curves USA, Piscataway, NJ, USA) instrument or in 15 ml glass tubes (not shown). Colony-forming units (CFU) counts after 16 h of growth were also assessed and correlated well to the growth curves (Fig 2D).

To avoid the effect of auto-aggregation and biofilm formation in the improved fitness of RB211 in imipenem, a mutation in the *pelA* gene was inserted into RB210 and RB211 strains. PelA is a protein involved in the synthesis of the Pel extracellular polysaccharide, which is essential for PA14 aggregation and biofilm formation, and thus its mutation made RB210 and RB211 unable to form biofilms (Supporting Information Fig. S3). Growth of *pelA* mutant strains overexpressing PvrR or PA14_72420 was monitored (Fig. 3) and results were similar to the wild-type background, thus ruling out the aggregation and polysaccharide roles in the growth rates observed due to altered c-di-GMP levels. The differences in growth rates both in *pelA* mutants and in the wild-type background were consistently reproducible and may provide a fitness advantage to the cells with higher c-di-GMP levels when grown together with cells that present less c-di-GMP.

Cells with higher c-di-GMP have a competitive advantage in the presence of imipenem

To test the hypothesis that higher c-di-GMP levels would confer a competitive advantage in the presence of sub-MIC imipenem, RB210, RB211 and PA14 cells were tagged with a cyan fluorescent protein (CFP) by introducing the *cfp* gene in the *att* Tn7 site. PA14*cfp* and RB211*cfp* strains presented the same growth behaviour as the unmarked parental cells, but RB210*cfp* had a growth deficiency (Supporting Information Fig. S4) and was not used further. Competition assays were performed in MHB using pairs of CFP-marked versus unmarked strains. Experiments performed in 15 ml tubes or in 96-well plates gave similar results. After 24 h of growth in 0.5 μg ml^{-1} of imipenem, RB211 cells

Table 1. Strains and plasmids used in this study.

Strains	Description[a]	Reference
PA14	Wild-type *P. aeruginosa* UCBPP-PA14	(Rahme *et al.*, 1995)
RB210	PA14 strain with *pvrR* gene under arabinose-inducible pBAD promoter control integrated in mini-CTX site.	This work
RB211	PA14 strain with *PA14_72420* gene under arabinose-inducible pBAD promoter control integrated in mini-CTX site.	This work
PA14cfp	Wild-type *P. aeruginosa* UCBPP-PA14 Tagged with CFP protein integrated in MiniTn7 site.	(Rahme *et al.*, 1995)
RB210*cfp*	RB210 tagged with CFP protein integrated in MiniTn7 site.	This work
RB211*cfp*	RB211 tagged with CFP protein integrated in MiniTn7 site.	This work
pelA::tn	PA14 strain with transposon in the *pelA* gene; Gm^R	(Liberati *et al.*, 2006)
RB210 pelA::tn	RB210 strain with transposon in the *pelA* gene; Gm^R	This work
RB211 *pelA::tn*	RB211 strain with transposon in the *pelA* gene; Gm^R	This work
tpbA::tn	PA14 strain with transposon in the *tpbA* gene; Gm^R	(Liberati *et al.*, 2006)
PA14_72420::tn	PA14 strain with transposon in the *PA14_72420* gene; Gm^R	(Liberati *et al.*, 2006)

Plasmid	Description	Reference
pJN105	*araC*-pBAD cloned in pBBR1MCS-5; Gm^R	(Newman and Fuqua, 1999)
Mini-CTX2	Contains *attP* site for integration at chromosomal *attB* site; Tc^R	(Hoang *et al.*, 1998)
pTNS3	Suicide helper expressing *tnsABCD* from P1 and Plac; Ap^R	(Choi *et al.*, 2005)
pUC18T-mini-Tn7T-Gm-ecfp	Gm^r on mini-Tn7T; mobilizable; for CFP tagging of Gm^s bacteria	(Choi and Schweizer, 2006; Choi *et al.*, 2006)
pFLP2	Ap^R, FLP recombinase expression plasmid	(Hoang *et al.*, 1998)
pGEM-T Easy	Cloning vector; Ap^R	Promega
pGGN01	*pvrR* coding region in pGEM-T Easy	This work
pGGN02	*PA14_72420* coding region in pGEM-T Easy	This work
pGGN03	1.2KB EcoRI-SpeI fragment of pGGN01 (*pvrR* coding region) into EcoRI-SpeI of pJN105; Gm^R	This work
pGGN04	2.3KB EcoRI-SpeI fragment of pGGN02 (*PA14_72420* coding region) into EcoRI-SpeI of pJN105; Gm^R	This work
pGGN05	2.5KB KpnI-SpeI fragment of pGGN03 (*araC*-pBAD and *pvrR* coding region) into EcoRI-KpnI of Mini-CTX2; Tc^R	This work
pGGN06	3.6KB KpnI-SpeI fragment of pGGN04 (*araC*-pBAD and *PA14_72420* coding region) into EcoRI-KpnI of Mini-CTX2; Tc^R	This work

a. Resistance markers: Gm^R, gentamycin; Ap^R, ampicillin; Tc^R, tetracyclin.

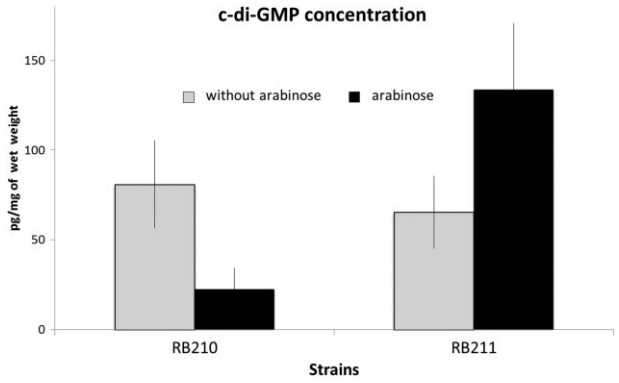

Fig. 1. Strains RB210 (low c-di-GMP levels) and RB211 (high c-di-GMP levels) have a sixfold difference in c-di-GMP intracellular concentration. Cells from 20 ml culture in minimal medium had their nucleotides extracted as described in the *Experimental procedures* section and analysed by HPLC-MS/MS. Results are presented from assays carried out in triplicates and error bars represent the standard deviation.

dominated both PA14 and RB210 cells and PA14 won the competition against RB210 cells; in other words, cells with a higher c-di-GMP concentration were predominant when co-cultured with cells with lower c-di-GMP levels. RB210 numbers are always smaller than the competitors in the presence of sub-MIC imipenem (Fig. 4A), probably because this strain has the lowest c-di-GMP content among all strains analysed and it may not be able to modulate its c-di-GMP content in response to the environmental conditions in a manner similar to the wild type. For example, RB211*cfp* corresponded to about 86% of the cells when co-cultivated with PA14 in imipenem, but PA14 and RB211*cfp* numbers were nearly the same in the absence of an antibiotic (Fig. 4B). Therefore, the apparent small differences in the growth rate in single strain cultures were reflected in superior fitness of cells carrying higher c-di-GMP levels in mixed cultures in the presence of the selective pressure.

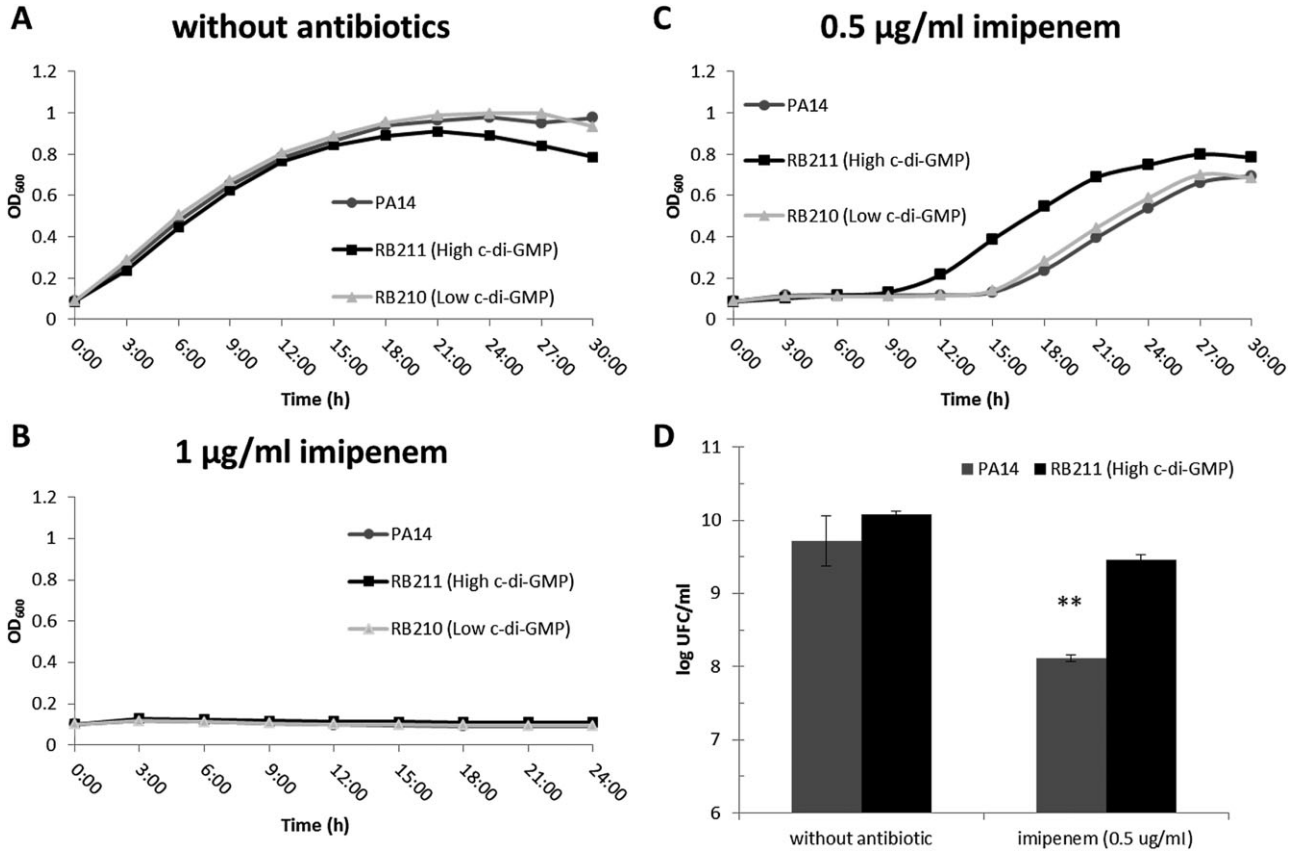

Fig. 2. High c-di-GMP levels lead to improved fitness in the presence of subinhibitory imipenem concentration. Strains with high (RB211) or low (RB210) c-di-GMP levels were grown in LB up to $OD_{600} = 1$, cultures were adjusted to $OD_{600} = 0.05$ into:
A. 300 µl MHB plus 0.2% arabinose without antibiotics.
B. 1 µg ml^{-1} imipenem.
C and D. 0.5 µg ml^{-1} imipenem.
Growth was monitored by Spectramax Paradigm (Molecular Devices) in a 48-well plate (A–C). After 16 h of growth in MHB or MHB plus 0.5 µg ml^{-1} of imipenem, cultures were serially diluted and plated for CFU counting (D). All measures were done in triplicates.

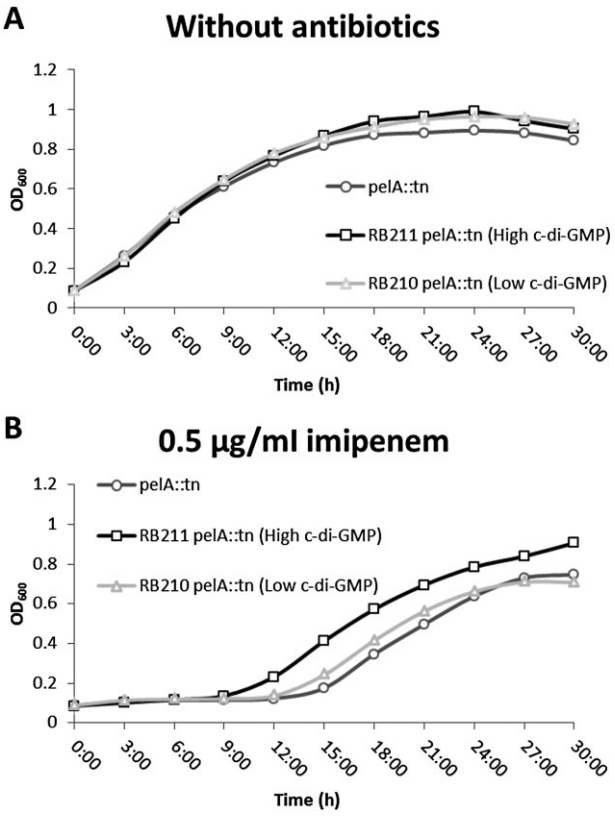

Fig. 3. Improved fitness of cells with high c-di-GMP levels is uncoupled to exopolysaccharide production. Strains with a biofilm defective background with high (RB211 *pelA::tn*) or low (RB210 *pelA::tn*) c-di-GMP levels were grown in LB up to $OD_{600} = 1$, cultures were adjusted to $OD_{600} = 0.05$ into:
A. 300 µl MHB plus 0.2% arabinose without antibiotics.
B. 0.5 µg ml^{-1} imipenem.
Growth was monitored at OD_{600} using Spectramax Paradigm (Molecular Devices) in a 48 well plate.

c-di-GMP alters porins levels but does not regulate the expression of resistance genes

To identify factors that could be involved in the improvement of antibiotic fitness at high c-di-GMP levels, we performed a proteomic analysis of RB210 and RB211 under induced conditions. Approximately 800 spots were assessed under each condition, with 29 proteins found to be differentially expressed in the high and low c-di-GMP strains ($P \leq 0.05$ and fold change cut-off ≥ 1.3) (Table 2). Interestingly, we found that five proteins underexpressed in the high c-di-GMP background strain were outer membrane porins (OprD, OprE, OprF, OprG and OpdC) (Fig. 5A, Table 2). Some proteins upregulated in the strain with high c-di-GMP levels (RB211) are putatively involved in antibiotic resistance mechanisms and include the protease-related chaperones HslU and ClpA and the endopeptidase PrlC (see discussion below). By using quantitative reverse transcription polymerase chain reaction (qRT-PCR), we analysed the mRNA levels of the five porin genes and the three protease-related proteins mentioned above in the high and low c-di-GMP backgrounds and found no differences, suggesting a possible role for post-transcriptional regulation mechanisms (Fig. 6A).

To analyse if there was transcriptional regulation of other mechanisms involved in antibiotic resistance, the relative mRNA levels of the β-lactamase *ampC* gene and of the six main efflux pumps genes (*mexA*, *mexC, mexE mexG, mexX* and *PA14_18760*) were determined in the RB210 and RB211 strains, and no differential expression was observed (Fig. 6B). Therefore, the altered fitness of the strains in the presence of imipenem is not due to increased expression of efflux pumps and β-lactamase. Therefore, it seems that c-di-GMP levels have no role in the transcriptional control of the genes analysed.

High c-di-GMP concentrations decrease OprD levels

The OprD, OpdC and OprE proteins belong to the OprD porin family, involved in transport of specific molecules, such as basic amino acids by OprD and histidine by OpdC (Hancock and Brinkman, 2002). Imipenem is a synthetic β-lactam that enters *P. aeruginosa* cells via the OprD porin (Kohler *et al*., 1999; Ochs *et al*., 1999). Absence of *oprD* makes *P. aeruginosa* up to 10 times more resistant to imipenem (Masuda *et al*., 1995). Western blot using a polyclonal antibody against OprD confirmed that the strain RB211 with high c-di-GMP levels has decreased OprD protein amounts and the opposite occurs with RB210, corroborating the 2D gel analysis (Fig. 5B). We also analysed a *tpbA* mutant that was previously shown to have high c-di-GMP levels (Ueda and Wood, 2009) and observed that this strain presents reduced OprD levels as well (Fig. 5B). Unexpectedly, the *PA14_72420* mutant did not show significant differences in OprD levels, perhaps because of compensation by other diguanylate cyclase proteins.

Discussion

c-di-GMP modulates the establishment of the biofilm mode of growth in many bacteria. The intracellular concentration of this molecule is the main factor directing the decision between the sessile and the planktonic lifestyles. It is already well established that the biofilm mode of growth increases the resistance to numerous antibiotics. Also, swarming cells, which have intracellular c-di-GMP concentrations lower than planktonic cells, are more resistant to a wide range of antibiotics (Lai *et al*., 2009). Both behaviours are related to intracellular c-di-GMP concentrations, which led us to speculate about the role of this molecule in controlling bacterial fitness in the presence of antibiotics. Although it seems intuitive, little is known about antibiotic resistance and c-di-GMP, apart

A

	Control			0.5 μg/ml imipenem		
CFP cells (%)	PA14	RB210	RB211	PA14	RB210	RB211
PA14cfp	ND	57 ± 1.3	47 ± 2.9	ND	73 ± 2.1	9.4 ± 2.3
RB211cfp	48 ± 1.2	53 ± 2.2	ND	86 ± 2.7	95 ± 3.2	ND

B

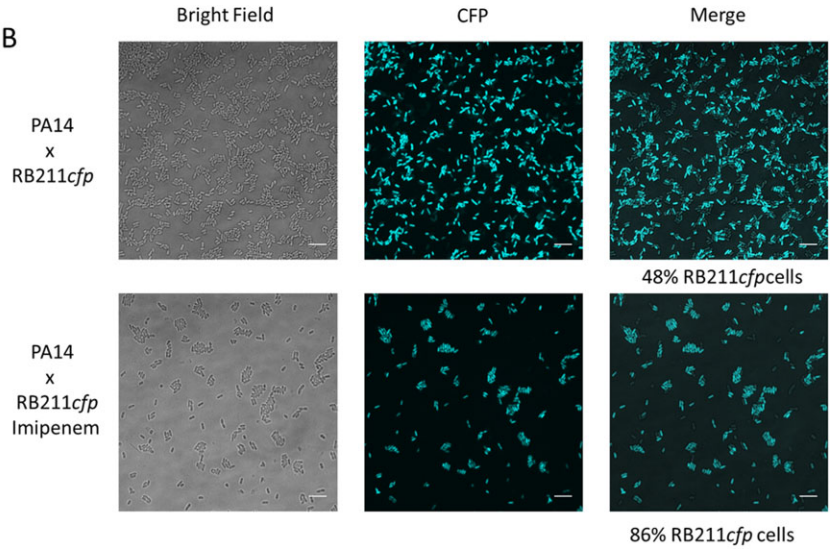

Fig. 4. High c-di-GMP levels lead to a competitive advantage in the presence of imipenem.
A. Pairs of strains were grown in MHB with or without 0.5 μg ml^{-1} of imipenem for 24 h. Two thousand total cells from each assay were counted under bright field microscopy and CFP labelled cells under a CFP BrightLine Filter Set. The percentage of fluorescent cells is shown.
B. The pictures represent one assay with RB211*cfp* (high c-di-GMP levels) and the wild-type strain. Bars represent 10 μm.

from its involvement in biofilm formation. In this report, we show that c-di-GMP has a role in *P. aeruginosa* fitness when grown at sub-MIC imipenem concentration. We also found that c-di-GMP affects the production of at least five porin proteins, which may alter the characteristics of the outer membrane and may account for the improved fitness. Whether this effect is direct or indirect remains to be determined.

Imipenem is a β-lactam antibiotic used in therapy for ventilator-associated pneumonia and commonly utilized in suspicion of *P. aeruginosa* infection (Torres, 2006). OprD is the protein responsible for imipenem uptake and lack of OprD renders the cells highly resistant to this antibiotic (Kohler *et al*., 1999; Ochs *et al*., 1999). Here we show that high c-di-GMP levels lead to a decrease in OprD protein levels and that a strain overexpressing a diguanylate cyclase exhibits improved growth in sub-MIC imipenem concentration. While it is tempting to correlate these two pieces of information, further experiments will be required to confirm this hypothesis. Numerous studies correlate OprD levels with β-lactam resistance but none has presented a mechanism by which the amount of OprD in the membrane is regulated post-transcriptionally. Interestingly, recent reports showed that *oprD* transcription is under the control of an extracytoplasmic function (ECF) sigma factor (Gicquel *et al*., 2013; Blanka *et al*., 2014).

The role of c-di-GMP in controlling extracytoplasmic proteases has been characterized (Kumagai *et al*., 2011; Navarro *et al*., 2011), and membrane and cytoplasmic proteases were associated with aminoglycoside resistance (Hinz *et al*., 2011). Our proteomic analysis revealed that the proteins HslU and ClpA, which are accessories to the proteases HslV and ClpP, are altered when varying the c-di-GMP levels. It is noteworthy that these proteases have been previously linked to antibiotic resistance (Hinz *et al*., 2011; Fernandez *et al*., 2012). Impermeability of the bacterial membrane is a well-known antibiotic resistance mechanism. While outer membrane proteins (Hancock, 1997; Breidenstein *et al*., 2011) and proteases (Hinz *et al*., 2011; Fernandez *et al*., 2012) are involved in antibiotic resistance mechanisms, there is no current model explaining how this works.

Only a few studies demonstrate a possible relationship of c-di-GMP in antibiotic adaptation in *P. aeruginosa*. Drenkard and Ausubel (2002) showed that *P. aeruginosa* PA14 grows in two different morphology types when plated in the presence of subinhibitory concentrations of kanamycin: wild-type smooth colonies, and a rough small colony variant often seen in biofilm-derived cells. PA14 overexpressing the PvrR phosphodiesterase plated under the same conditions presents only the wild-type phenotype. Hoffman and colleagues (2005) showed that biofilm formation is induced, when the PAO1 strain is incubated under subinhibitory tobramycin concentrations and that the Arr phosphodiesterase is involved in this event. Recently, it has been demonstrated that *P. aeruginosa* cells with low c-di-GMP levels are more resistant to the antimicrobial peptide colistin than cells with high c-di-GMP levels (Chua *et al*., 2013). These data, together with what we present here, show that c-di-GMP is involved in

Table 2. Differentially expressed proteins due to varying c-di-GMP levels.

Outer membrane porins			
PA14 ID	PAO1 ID	High/low c-di-GMP ratio	Predicted function
PA14_02020	PA0162	0.59	Outer membrane porin OpdC, OprD family
PA14_03800	PA0291	0.58	Anaerobically-induced outer membrane porin OprE
PA14_11270	PA4067	0.54	Outer membrane protein OprG precursor
PA14_41570	PA1777	0.71	Major porin and structural outer membrane porin OprF
PA14_51880	PA0958	0.51	Basic amino acid, basic peptide and imipenem outer membrane porin OprD
Other proteins			
PA14 ID	PAO1 ID	High/low c-di-GMP ratio	Predicted function
PA14_11550	PA4044	0.63	1-deoxy-D-xylulose-5-phosphate synthase
PA14_16090	PA3735	0.65	Threonine synthase
PA14_16510	PA3701	0.73	Putative peptide chain release factor 2
PA14_21230	PA3308	0.62	ATP-dependent helicase HepA
PA14_41360	PA1795	0.73	Cysteinyl-tRNA synthetase
PA14_41380	PA1794	0.79	Glutaminyl-tRNA synthetase
PA14_50290	PA1092	0.75	Flagellin type B
PA14_62690	PA4739	0.60	Hypothetical protein
PA14_00790	PA0067	1.52	Oligopeptidase A (PrlC)
PA14_05620	PA0432	1.86	S-adenosyl-L-homocysteine hydrolase
PA14_11690	PA4031	1.64	Inorganic pyrophosphatase
PA14_14730	PA3814	1.63	Cysteine desulfurase
PA14_17070	PA3655	1.61	Elongation factor Ts
PA14_25690	PA2965	1.31	3-oxoacyl-(acyl carrier protein) synthase II
PA14_30230	PA2620	1.82	ATP-dependent Clp protease, ATP-binding subunit ClpA
PA14_42690	PA1687	1.81	Spermidine synthase
PA14_57710	PA4442	1.55	Bifunctional sulfate adenylyltransferase subunit 1/adenylylsulfate kinase protein
PA14_57810	PA4450	1.44	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
PA14_57890	PA4457	2.65	Sugar phosphate isomerase KdsD
PA14_62710	PA4740	1.33	Polynucleotide phosphorylase/polyadenylase
PA14_64110	PA4848	1.67	Acetyl-CoA carboxylase biotin carboxylase subunit
PA14_65190	PA4936	1.65	TrmH family RNA methyltransferase, group 3
PA14_66790	PA5054	1.57	ATP-dependent protease ATP-binding subunit HslU
PA14_67490	PA5110	1.20	Fructose-1,6-bisphosphatase

P. aeruginosa adaptation when exposed to subinhibitory concentration of antibiotics. Because drugs such as β-lactams, aminoglycosides and antimicrobial peptides act through diverse mechanisms, it is not surprising that resistance to different classes of drugs may be favoured by contrasting c-di-GMP levels and that there are still many questions to be addressed on this subject. One of the mechanisms that might contribute to adaptation in the presence of antibiotics is changing the outer membrane profile, which may alter the way cells interact with the external milieu and with one another, enabling them to cope with the presence of potentially harmful compounds.

Experimental procedures

Strains construction and phenotype analysis

Strains RB210 and RB211 were constructed as follows: coding regions of *pvrR* (RB210) or *PA14_72420* (RB211) genes were amplified and cloned in pGEM-T Easy (Promega, Madison, WI, USA), generating pGGN01 and pGGN02 plasmids respectively. A 1.2 kb EcoRI-SpeI fragment of pGGN01 and a 2.3 kb EcoRI-SpeI fragment of pGGN02 were cloned downstream of the pBAD promoter into pJN105 plasmid (Newman and Fuqua, 1999), generating pGGN03 and pGGN04 plasmids respectively. Promoter–gene fusions and the *araC* gene were excised from pGGN03 and pGGN04 by KpnI-SpeI digestion and cloned into the mini-CTX2 plasmid (Hoang *et al.*, 2000). The resulting pGGN05 and pGGN06 plasmids (Table 1) were transferred by conjugation *P. aeruginosa* PA14 strain and integrated in the PA14 genome in the *ctx* site. The plasmid backbone was then cured from the chromosome of each transconjugant using the pFLP2-encoded Flp recombinase (Hoang *et al.*, 1998) to leave only the *araC*-pBAD *pvrR/PA72420* fusion in the chromosome. Plasmid pFLP2 was introduced into *P. aeruginosa* via electroporation, and carbenicillin-resistant colonies were cultivated in the absence of tetracycline to allow recombination of the FRT sites, and subsequently, samples of the liquid cultures containing carbenicillin-resistant colonies were streaked onto L-agar containing 10% (weight/volume) sucrose to select for the loss of plasmid pFLP2 (following excision of the mini-CTX backbone from the chromosome). Sucrose-resistant colonies were patched on L-agar plates containing tetracycline (25 μg ml^{-1}) or carbenicillin (100 μg ml^{-1}) to confirm the loss of the mini-CTX backbone (tetracycline sensitive) and the pFLP2 plasmid (carbenicillin sensitive). Construction of PA14*cfp*, RB210*cfp* and RB211*cfp* was performed by electroporation of pUC18T-mini-Tn7T-Gm-ecfp as described by Choi and Schweizer (2006). The plasmid backbone (with antibiotic-resistance

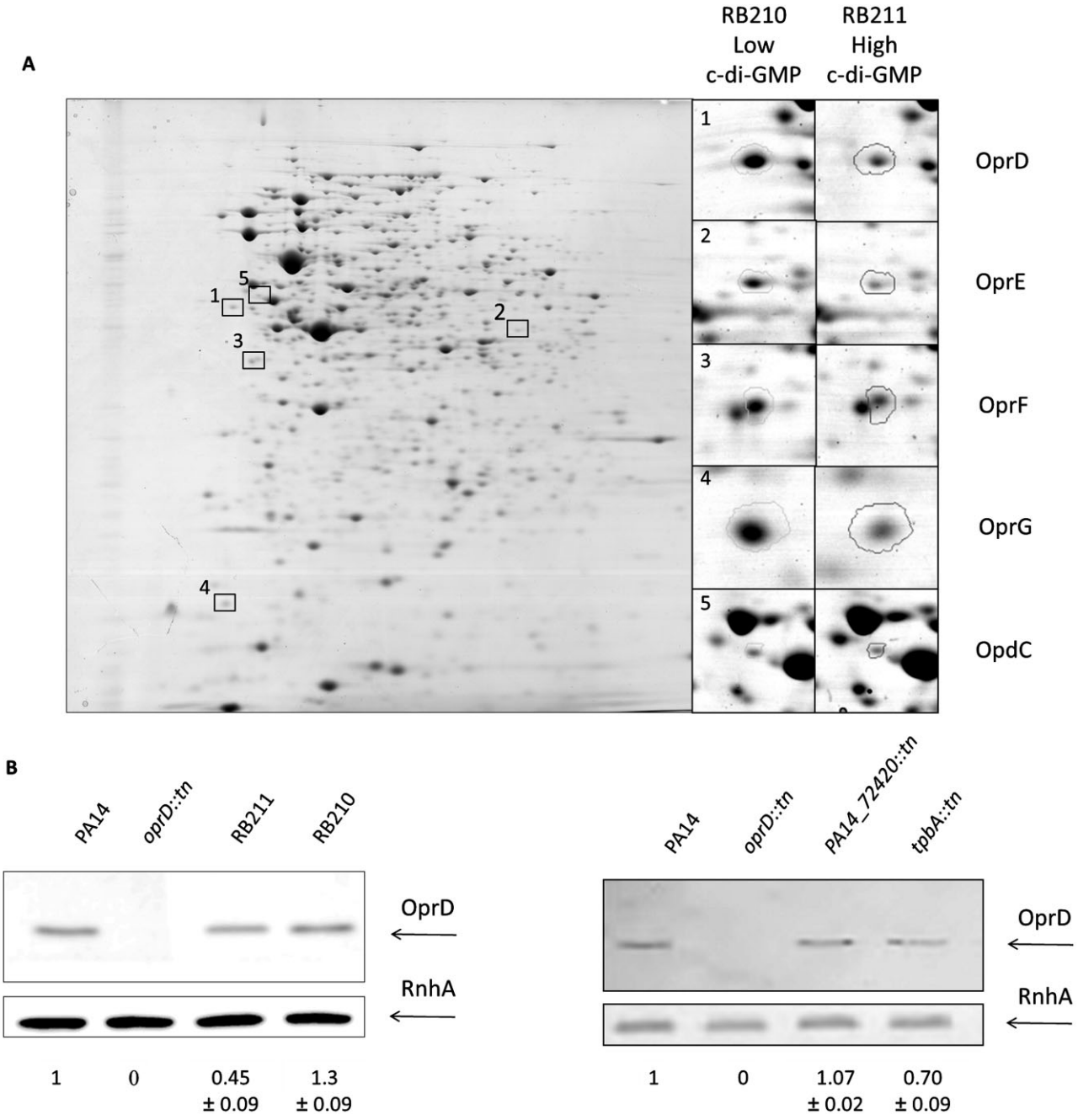

Fig. 5. A. Altered porins levels in strains having opposite c-di-GMP levels. Cellular proteins were extracted from strains with high (RB211) and low (RB210) c-di-GMP levels strains grown in LB with 0.2% arabinose to $OD_{600} = 1$. The analysis were done with biological triplicates in 2D gels with 3–10 non-linear pH gradients and 12% acrylamide in the second dimension, using Delta 2D software (Decodon, Greifswald, Germany). Numbered boxes show selected sections of 2D gels with outer membrane proteins in high and low c-di-GMP levels proteomes outlined and indicated at the right side. ND, no data.
B. Western blot analysis of the OprD protein. Cellular proteins were extracted from cells grown with 0.2% arabinose to $OD_{600} = 1$. Anti-RNase HI (RnhA) was used as loading control. The numbers below the figure represent the relative ratio of OprD/RnhA, performed with ImageJ software, considering PA14 as 1. Panels shown are representative of three independent experiments.

gene) was removed by Flp recombinase as described above. The RB210 *pelA::tn* and RB211 *pelA::tn* were constructed by electroporation of 1 μg of genomic DNA of *pelA::tn* strain obtained from the PA14NR library (Liberati *et al.*, 2006), followed by selection in Luria–Bertani (LB) plates containing 5 μg ml^{-1} of gentamycin. The transconjugant strains had the *pelA* gene sequenced to confirm the mutation. All strains used in this work are described in Table 1.

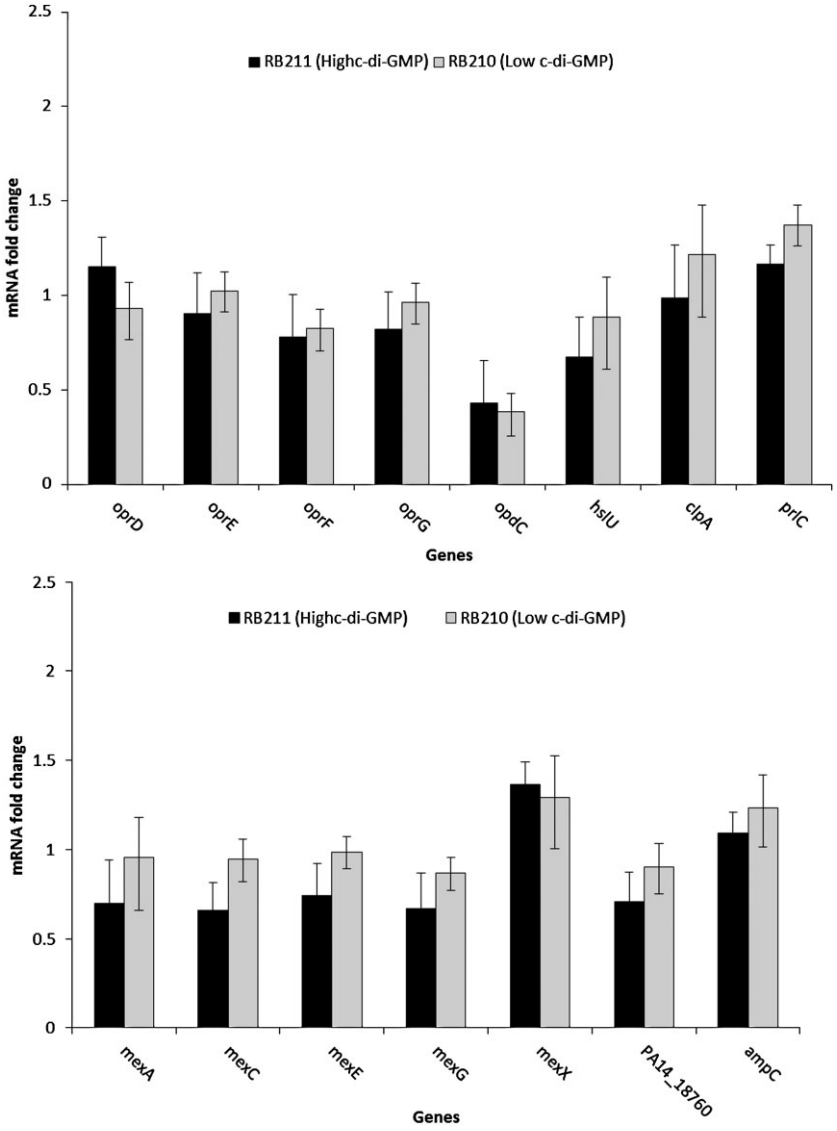

Fig. 6. c-di-GMP concentration has no effect on mRNA levels of genes related to antibiotic resistance. Different c-di-GMP levels have no significant effect in the transcription of porins, protease-related chaperones, *prlC*, efflux pumps and *ampC* genes. Total RNA was extracted with Trizol when cultures reached O.D600 = 1.0, treated with DNase I and used for cDNA synthesis. cDNA was amplified with specific primers using SYBR Green PCR Master Mix in the 7300 Real Time PCR System (Applied Biosystems). The threshold cycle data analysis was employed, with *nadB* used for normalization of cDNA amount.

Biofilm assay

Cultures in stationary phase were diluted to $OD_{600\ nm} = 0.05$ in LB broth with or without arabinose, and were incubated at 30°C for 16 h in glass tubes or 96-well polystyrene plates. Initial bacterial adhesion was observed by staining the biomass by gently pouring off the media, washing twice with water and adding 0.1% crystal violet. Tubes were washed and rinsed with water until all unbound dye was removed. Three independent assays were carried out for each strain.

Motility assays

Swarming assays were performed as described previously by O'Toole and colleagues (1999) and Tremblay and Déziel (2008). Swarm plates were incubated at 30°C for 16 h. Swimming assays were performed by inoculating 5 μl of a stationary phase-grown liquid cultures in T medium plates (Triptone 10 g l^{-1}; NaCl 5 g l^{-1}) 0.3% agar that were incubated for 16 h at 30°C in a plastic bag to maintain the humidity constant.

Isolation and detection of c-di-GMP

c-di-GMP was extracted as described by Simm and colleagues (2004) with slight modifications. Bacteria were cultivated overnight in M63 2% glucose medium at 37°C with shaking. Cells were harvested (20 ml culture volume) by quick centrifugation at 4°C, the resulting pellets were re-suspended in 300 μl of water, heated at 100°C for 5 min and nucleotides were extracted twice with 0.5 ml of 70% ice-cold ethanol. The extract was lyophilized, re-suspended in 300 μl of water and 100 μl were subjected to HPLC-MS/MS analysis as described by Spangler and colleagues (2010).

Proteomic analysis. Bacterial strains were grown in LB medium to reach an $OD_{600} = 1$, washed twice and

re-suspended in a 2D extraction buffer (7 M urea/2 M thiourea/2% CHAPS/40 mM DDT and protease inhibitors), centrifuged twice at 18 000 × *g* for 60 min at 4°C. Protein concentration was determined with the Bradford method (Sigma-Aldrich, St. Louis, MO, USA). Protein extracts (350 μg) were suspended in 2D running buffer (7 M urea/2 M thiourea/2% CHAPS/40 mM DDT/2% Pharmalite and 10% glycerol), separated in the first dimension according to their isoelectric point by isoelectric focusing (IEF), and then resolved in the second dimension, according to molecular weight by SDS-PAGE. IEF was performed by using immobilized pH gradient strips with a nonlinear pH range from 3 to 10 on IPGphor III with a programmed voltage gradient. SDS-PAGE was performed on a 12.5% polyacrylamide gel. Gels were stained with colloidal coomassie blue and analysed with the DECODON Delta 2D software. Spots were considered differentially expressed when *t*-test generated $P < 0.05$ values. In-gel digestion was performed as described by Hellman and colleagues (1995). The tryptic peptides were eluted from the gel with acetonitrile containing 0.1% of trifluoro-acetic acid and separated on an Agilent Nanopump using a C18 ZORBAX trap and a SB-C18 ZORBAX 300 reversed phase column (150 mm × 75 μm, 3.5 μm particle size) (Agilent Technologies, Santa Clara, CA, USA). All mass spectra were recorded on a hybrid linear ion trap–triple quadrupole mass spectrometer (Q-Trap, AB Applied Biosystems/MDS SCIEX Instruments, Foster City, CA, USA) equipped with a nano-electrospray ionization source. The accumulation of MS-MS data was performed with the Analyst Software, version 1.4 (AB Applied Biosystems/MDS SCIEX Instruments). MASCOT (Matrix Science, London, UK) was used to create peak lists from MS and MS/MS raw data.

Western blot analysis. A polyclonal antiserum was obtained by inoculating mice with His-tagged OprD. An anti-RNase HI antiserum was used to normalize the protein amounts in each lane because this protein does not vary in several growth conditions (Meireles, 2011). The blots were probed with primary antibodies incubated overnight at 4°C and the immunopositive bands were visualized by using ECL Plex Western Blotting Detection Reagents (GE Healthcare, Fairfield, CT, USA). Densitometry analyses were performed using ImageJ software.

MIC determination, growth curves and CFU counts

Imipenem MIC were determined for PA14 and strains with altered c-di-GMP levels that were grown in LB up to $OD_{600} = 1$, then cultures were adjusted to $OD_{600} = 0.05$ into 200 μl cation-adjusted Mueller-Hinton broth (MHB) (Wiegand *et al.*, 2008) with 0.2% arabinose with serially diluted imipenem concentrations ranging from 0.125 to 16 μg ml^{-1}, incubated at 37°C at 250 r.p.m. and absence of growth was evaluated after 20 h.

For growth curves, cultures were grown in LB up to $OD_{600} = 1$ and adjusted to $OD_{600} = 0.05$ into 150 μl (96-well plates) or 500 μl (48-well plates) MHB with 0.2% arabinose, with or without imipenem at different concentrations. The growth curves were monitored as OD_{600}, each 15 min, with SpectraMax Paradigm (Molecular Devices, Sunnyvale, CA, USA) at 37°C with low orbital agitation. In the curves presented (Figs. 2, 3 and Supporting Information Fig. S3), the 15 min markers were excluded for clarity and they were shown for intervals of 3 h time. Experiments were repeated at least three times. Each time, from three to five wells were used for each condition and results are shown as average from a representative experiment.

For CFU counts, cultures were grown in triplicates in 15 ml glass tubes in MHB with or without imipenem, incubated at 37°C at 250 r.p.m. for 16 h. Samples were serially diluted and plated in LB–agar.

Fluorescence microscopy

Microscopy was performed on a Nikon Eclipse TiE microscope, with filters for CFP (CFP BrightLine Filter Set) and a Plan APO VC Nikon 100 objective lens (oil, NA 1.4) (Tokyo, Japan). Images were captured with an Andor I-Xon EMCCD camera (Belfast, NIR, UK) and the images were processed and analysed with Nikon NIS Elements software (version 3.07) and ImageJ (http://rsb.info.nih.gov/ij/). The cells were grown to specific assay condition and the microscopy assays were performed as described by dos Santos and colleagues (2012).

qRT-PCR

For qRT-PCRs, total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA), treated with DNase I (Thermo Scientific, Waltham, MA, USA) and used for cDNA synthesis with Improm II (Promega, Madison, WI, USA) or Superscript III (Invitrogen) and hexamer random primers (Thermo Scientific). cDNA was then amplified with specific primers using Maxima SYBRGreen/ROX qPCR Master Mix (Thermo Scientific) and the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). *nadB* was used as internal control for normalization of total RNA levels (Lequette *et al.*, 2006). The relative efficiency of each primer pair was tested and compared with that of *nadB* and the threshold cycle data analysis ($2^{-\Delta\Delta Ct}$) was used (Livak and Schmittgen, 2001). All reactions were performed in triplicates, the assays were repeated at least twice using independent cultures and the results of one representative experiment are shown, with average values of technical triplicates and error bars representing standard deviation of ΔΔCt.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Altered c-di-GMP levels in strains RB210 and RB211 affect biofilm formation and motility. For motility assays, 0.3% agar T medium (swimming) and 0.5% agar M9 medium (swarming) plates were inoculated with 5 ml of a culture at $OD_{600} = 3$ and incubated for 16 h at 30°C inside plastic bags to maintain humidity. To assess biofilm formation, bacteria were inoculated in LB at $OD_{600} = 0.01$ in glass tubes and kept at 30°C for 16 h without agitation. The medium was discarded and adhered cells washed and stained with 1% crystal violet.
Fig. S2. High c-di-GMP results in improved growth when cultures are grown at sub-MIC imipenem concentrations. Strains with altered c-di-GMP levels were grown in LB up to $OD_{600} = 1$, then cultures were adjusted to $OD_{600} = 0.05$ into 200 μl of MHB plus 0.2% arabinose with serial diluted imipenem concentrations. MIC was evaluated after 20 h of growth.
Fig. S3. RB210*pelA::tn* and RB211*pelA::tn* strains do not form biofilms. A mutation in the *pelA* gene was inserted by

marker exchange in the chromosome of RB210 and RB211 strains, as described in the *Experimental procedures* section. Biofilm assays were performed as described in the legend of Supporting Information Fig. S1.

Fig. S4. *cfp* tagging does not affect the growth of wild-type and high c-di-GMP level cells. Strains with high (RB211) or low c-di-GMP (RB210) levels in a *cfp*$^+$ background were grown in LB up to $OD_{600} = 1$, cultures were adjusted to $OD_{600} = 0.05$ in MHB plus 0.2% arabinose and 0.5 μg ml^{-1} of imipenem. Samples of 300 μl were poured in triplicates in a 48-well plate and growth was monitored as described in *Experimental procedures*.