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Tyrosine phosphatase TpbA of *Pseudomonas aeruginosa* controls extracellular DNA via cyclic diguanylic acid concentrations

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Summary

Inactivating the tyrosine phosphatase TpbA of *Pseudomonas aeruginosa* PA14 induces biofilm formation by 150-fold via increased production of the second messenger cyclic diguanylic acid (c-di-GMP). Here, we show the *tpbA* mutation reduces extracellular DNA (eDNA) and that increased expression of *tpbA* increases eDNA; hence, eDNA is inversely proportional to c-di-GMP concentrations. Mutations in diguanylate cyclases PA0169, PA4959 and PA5487 and phosphodiesterase PA4781 corroborate this trend. The *tpbA* mutation also decreases cell lysis while overexpression of *tpbA* increases cell lysis. To further link c-di-GMP concentrations and eDNA, the gene encoding phosphodiesterase PA2133 was overexpressed which increased eDNA and decreased biofilm formation by decreasing c-di-GMP. Furthermore, the effect of the *tpbB* mutation along with the *tpbA* mutation was examined because loss of TpbB restored the phenotypes controlled by enhanced c-di-GMP in the *tpbA* mutant. The *tpbA tpbB* double mutations restored eDNA to that of the PA14 wild-type level. These findings suggest that c-di-GMP, rather than TpbA, controls eDNA. Hence, TpbA acts as a positive regulator of eDNA and cell lysis by reducing c-di-GMP concentrations.

Introduction

Biofilms are a complex community in which bacteria live and communicate with each other. Biofilms formed by pathogenic bacteria often cause chronic infectious diseases (Davies, 2003), because bacteria in biofilms are more resistant to antibiotics (Davies, 2003). *Pseudomonas aeruginosa*, an opportunistic pathogen, is persistent during cystic fibrosis infections because of its ability to form biofilms (Ryder *et al*., 2007). Hence, to control bacterial biofilm formation, much effort has been directed toward inhibition of biofilm formation or stimulation of biofilm dispersal (Ren *et al*., 2001; Davies and Marques, 2009).

Bacterial biofilms consist of extracellular polysaccharides (EPS), nucleic acids and proteins (Ryder *et al*., 2007). In *P. aeruginosa*, the genetic loci responsible for EPS production are *psl* and *pel*; mutants lacking either *psl* or *pel* are dramatically inhibited in biofilm formation (Friedman and Kolter, 2004; Jackson *et al*., 2004). Production of EPS is controlled by the internal messenger, 3,5-cyclic diguanylic acid (c-di-GMP), which controls the switch from motility (low c-di-GMP) to sessility (high c-di-GMP) and controls virulence (Cotter and Stibitz, 2007). c-di-GMP concentrations are modulated directly via *de novo* synthesis by diguanylate cyclases (proteins with a GGDEF motif) and via degradation by phosphodiesterases (proteins with an EAL or HD-GYP motif) (Kulasakara *et al*., 2006; Pesavento and Hengge, 2009; Ryan *et al*., 2009). High c-di-GMP concentrations induce expression of genes related to EPS production (*pel* and *psl*) (Hickman *et al*., 2005; Ueda and Wood, 2009), directly activate activity of the Pel protein (Lee *et al*., 2007), and repress genes related to flagella in *P. aeruginosa* (Ueda and Wood, 2009). Subsequently, biofilm formation is enhanced by high concentrations of c-di-GMP and EPS, and is enhanced by reduced motility.

Extracellular DNA (eDNA) is a major component of the biofilm matrix (Whitchurch *et al*., 2002). DNase I treatment effectively inhibited biofilm formation by *P. aeruginosa* at the initial stage, but not at later stages (Whitchurch *et al*., 2002). eDNA is also important for the biofilm formation of Gram-positive bacteria, such as *Enterococcus faecalis* (Thomas *et al*., 2008), *Bacillus cereus* (Vilain *et al*., 2009) and *Staphylococcus epidermidis* (Qin *et al*., 2007). In *E. faecalis* and *S. epidermidis*, autolysis-mediated eDNA release contributes to forming the biofilm matrix (Qin *et al*., 2007; Thomas *et al*., 2008). Holin (CidA)–antiholin (LrgAB) control cell lysis in *Staphylococcus aureus* and subsequently affect biofilm maturation and tower formation (Mann *et al*., 2009). Thus, cell lysis causes eDNA release which alters the biofilm structure in microbial communities.

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Previously, we found that loss of TpbA, a tyrosine phosphatase, increases biofilm formation by 150-fold as well as increases EPS production by 28-fold most likely through its dephosphorylation of diguanylate cyclase TpbB (Ueda and Wood, 2009). These phenotypes were the result of elevated c-di-GMP concentrations in the *tpbA* mutant (Ueda and Wood, 2009). Here, we report for the first time the role of c-di-GMP on the production of eDNA. The *tpbA* mutant, which accumulates c-di-GMP, had 10-fold less eDNA compared with the wild-type strain. Corroborating this result, overexpression of either *tpbA* or PA2133 (encoding a phosphodiesterase), both of which reduce cellular c-di-GMP levels, increased eDNA. Loss of TpbB (a diguanylate cyclase), which reduces c-di-GMP, increased eDNA production by the *tpbA* mutant to that of the wild-type strain. We also show that other active GGDEF proteins (PA0169, PA4959 and PA5487) and HD-GYP-bearing PA4781 participate in the regulation of eDNA release via c-di-GMP. Therefore, c-di-GMP is a negative regulator of eDNA from *P. aeruginosa*.

Results and discussion

Strains used in this study are listed in Table S1, and both *P. aeruginosa* (Liberati *et al*., 2006) and *Escherichia coli* were grown in Luria–Bertani (LB) medium at 37°C. Gentamicin (15 μg ml^{-1}) and tetracycline (75 μg ml^{-1}) were used for growth of the *P. aeruginosa* transposon mutants, and carbenicillin (300 μg ml^{-1}) was used to maintain plasmids in *P. aeruginosa*. Ampicillin (100 μg ml^{-1}) was used with LB plates for growing *P. aeruginosa* to examine colony-forming units (cfu) as this strain is naturally resistant to ampicillin.

TpbA influences eDNA via c-di-GMP

Extracellular DNA has not been studied previously with the *tpbA* mutant. Here, eDNA in the supernatant of the *tpbA* culture was determined by quantitative polymerase chain reaction (qPCR). To minimize the effect of cell growth, the amount of eDNA was normalized by the total DNA amount which consists of eDNA in the supernatant and genomic DNA (gDNA) from unlysed cells.

To validate this assay, the *lasR rhlR* double mutant (Park *et al*., 2005) was used as a control because quorum sensing stimulates eDNA release (Allesen-Holm *et al*., 2006). In our assay, the *lasR rhlR* mutant released 50% less eDNA and this is comparable to the previous report, although the eDNA quantification method was different (Allesen-Holm *et al*., 2006).

By qPCR, we found that the *tpbA* mutant, which produces more c-di-GMP (Ueda and Wood, 2009), released 10-fold less eDNA than the wild-type strain (Fig. 1). eDNA was increased (restored) to that of the wild-type strain in the *tpbA tpbB* double mutant due to loss of TpbB function, which regulates biofilm formation downstream of TpbA (Ueda and Wood, 2009). A single mutation in *tpbB* did not affect eDNA levels (Fig. 1).

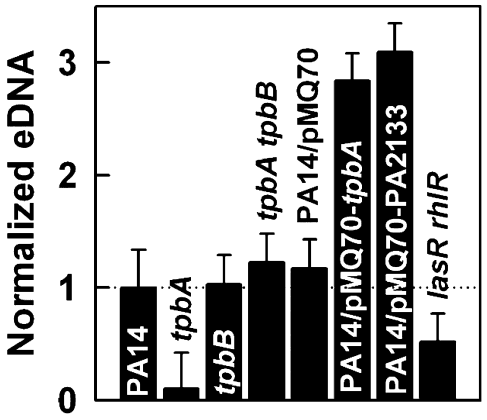

Fig. 1. c-di-GMP controls eDNA. eDNA released via qPCR after growth in LB at 37°C for 14 h. The *lasR rhlR* mutant was used as a control for reduced eDNA. eDNA was normalized by the total DNA (eDNA + gDNA) in the culture. Samples from cultures (1.5 ml) were centrifuged at 13 000 *g* for 10 min, and 900 μl of the supernatant was extracted with 900 μl of phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant (700 μl) was extracted again with 700 μl of chloroform, and nucleic acids were precipitated overnight with 50 μl of 3 M sodium acetate (pH 5.3) and 500 μl of isopropanol. Samples were centrifuged at 13 000 *g* for 10 min and the pellets were dissolved in 100–200 μl of TE with 20 μg ml^{-1} RNase A. To normalize eDNA, total DNA was quantified from 900 μl of culture by sonicating at 10 W for 1 min. qPCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). eDNA and total DNA (eDNA + gDNA) were quantified using the *pvdS* gene (primers shown in Table S2). For the calibration curve, PA14 gDNA was purified using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) and quantified using a UV spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). gDNA (10 pg to 10 ng) was used to prepare the calibration curve. At least two independent cultures were used for each strain (biological replicates), and two to three replicates were tested for each sample by qPCR (technical replicates); one standard deviation is shown.

Other c-di-GMP-related proteins influence eDNA

To validate that TpbA influences eDNA via c-di-GMP, we investigated eDNA upon expressing EAL protein PA2133 which degrades c-di-GMP (Kulasakara *et al*., 2006). PA14-36990 (orthologue of PA2133) was added to pMQ70 (Shanks *et al*., 2006) using the NheI and HindIII sites. PA14-36990 was amplified using Pfu DNA polymerase with primers PA14-36990-F-XbaI and PA14-36990-R-HindIII (Table S2). The resulting plasmid, pMQ70-PA2133, was transformed into PA14 and the *tpbA* mutant by conjugation (Ueda and Wood, 2009).

We confirmed the PA2133 protein is active in PA14 by testing biofilm formation; expression of PA2133 abolished biofilm formation in the wild-type strain similar to expressing *tpbA*, and there was a dramatic decrease in biofilm

formation in the *tpbA* mutant (Fig. S1). In addition, although the *tpbA* mutant is highly aggregative upon adding fresh medium (Ueda and Wood, 2009), expression of PA2133 restored aggregation of the *tpbA* mutant (data not shown).

Expression of both *tpbA* and PA2133 in PA14 increased eDNA dramatically (threefold, Fig. 1). Since both TpbA and PA2133 proteins reduce cellular c-di-GMP concentrations, we conclude again that eDNA is inversely proportional to cellular c-di-GMP levels.

To provide additional evidence that c-di-GMP levels impact eDNA, we also investigated whether a single mutation in several GGDEF/EAL/HD-GYP genes affects eDNA formation. To examine the effect of loss of a GGDEF protein (which reduces cellular c-di-GMP) on eDNA, the PA0169, PA0847, PA1107, PA1727, PA4959 and PA5487 mutants were tested because these proteins are active (a mutation in these genes causes abolished or decreased biofilm formation, and/or *in vitro* diguanylate cyclase activity was detectable) (Kulasakara *et al*., 2006).

Similarly, PA2133 and PA4367 were chosen as active EAL proteins (these mutants accumulate more c-di-GMP) (Kulasakara *et al*., 2006) and PA4108 and PA4781 were chosen as active HD-GYP proteins (Ryan *et al*., 2009). Of these 10 proteins, the PA0169 and PA5487 GGDEF mutants showed the largest increase in eDNA (Fig. 2). Both mutants were not able to form biofilm (Kulasakara *et al*., 2006); hence, these proteins may be responsible for production of a large portion of cellular c-di-GMP. Similarly, eDNA was increased upon deleting GGDEF protein PA4959, and eDNA was decreased slightly upon deleting HD-GYP protein PA4781 (Fig. 2). There was little impact on eDNA by mutating PA0847, PA1107, PA1727, PA2133, PA4367 and PA4108. Taken together, these results corroborate that eDNA is inversely proportional to c-di-GMP levels.

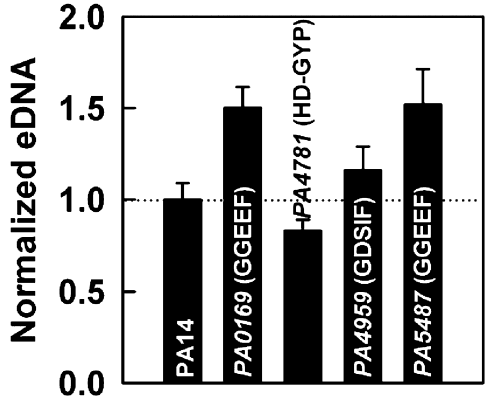

Fig. 2. Diguanylate cyclase and phosphodiesterase activities and eDNA. Mutations in PA0169 (PA14-02110), PA4781 (PA14-63210), PA4959 (PA14-65540) and PA5487 (PA14-72420), encoding either an active diguanylate cyclase or phosphodiesterase, were tested for their effect on eDNA after growth in LB at 37°C for 14 h. Normalized eDNA was quantified by qPCR with the *pvdS* primers and was calculated by the ratio of eDNA/total DNA. Data are the average of two independent cultures ± SD.

TpbA influences cell lysis

Cell lysis is often associated with eDNA in bacterial communities (Rice *et al*., 2007). In order to investigate whether c-di-GMP controls eDNA through cell lysis in *P. aeruginosa*, cell lysis was monitored by measuring turbidity at 600 nm, by counting cfu, by measuring extracellular total protein and by live/dead staining. Because of the aggregative phenotype of the *tpbA* mutant, all bacterial cultures were mildly sonicated to separate aggregated cells. After expressing *tpbA* (which should decrease c-di-GMP), turbidity began to decrease after 10 h (Fig. 3A), the same time at which eDNA was found to be released (Fig. 3C).

To evaluate bacterial growth more accurately, cfu were determined for each strain at 14 h. The *tpbA* mutant formed 1.5-fold more colonies than wild-type PA14 ($2.5 \pm 0.5 \times 10^{10}$ for PA14 wild-type and $3.8 \pm 0.4 \times 10^{10}$ for the *tpbA* mutant) (Fig. 3B). In contrast, production of TpbA reduced cfu by 5.7-fold compared with the wild-type strain ($3.4 \pm 0.3 \times 10^{9}$ for PA14/pMQ70 and $0.6 \pm 0.4 \times 10^{9}$ for PA14/pMQ70-*tpbA*) (Fig. 3B) which corroborates the decrease in turbidity. Therefore, production of TpbA lysed cells.

To corroborate these lysis results, extracellular total protein concentrations were measured as an indicator of cell lysis. Both supernatant samples and samples of the whole were obtained from bacterial cultures grown for 14 h; the whole bacterial culture samples were sonicated at 5 W for 90 s to lyse the cells. Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Extracellular total protein concentrations were normalized by dividing by total protein concentrations from both the supernatant and cells. Similar trends of extracellular total protein compared with cell growth and cfu were found with PA14 and the *tpbA* mutant; deletion of *tpbA*, which led to increased c-di-GMP, resulted in less cell lysis (Table S3). Live/dead staining was also tested to validate whether cell lysis occurs more in PA14 relative to the *tpbA* mutant. The results show that the *tpbA* mutation results in 2.4-fold more live cells at 14 h (Table S3). Together these three assays of cell viability indicate that TpbA increases cell lysis and eDNA by decreasing c-di-GMP.

These observations were also confirmed by the agarose gel electrophoresis method (Fig. 3C). Time-course analysis of eDNA showed that eDNA increases with culture age for PA14, the *tpbB* mutant and the *tpbA tpbB* mutant, but not for the *tpbA* mutant (Fig. 3C). Together, these findings indicate that higher concentrations of c-di-GMP reduce eDNA.

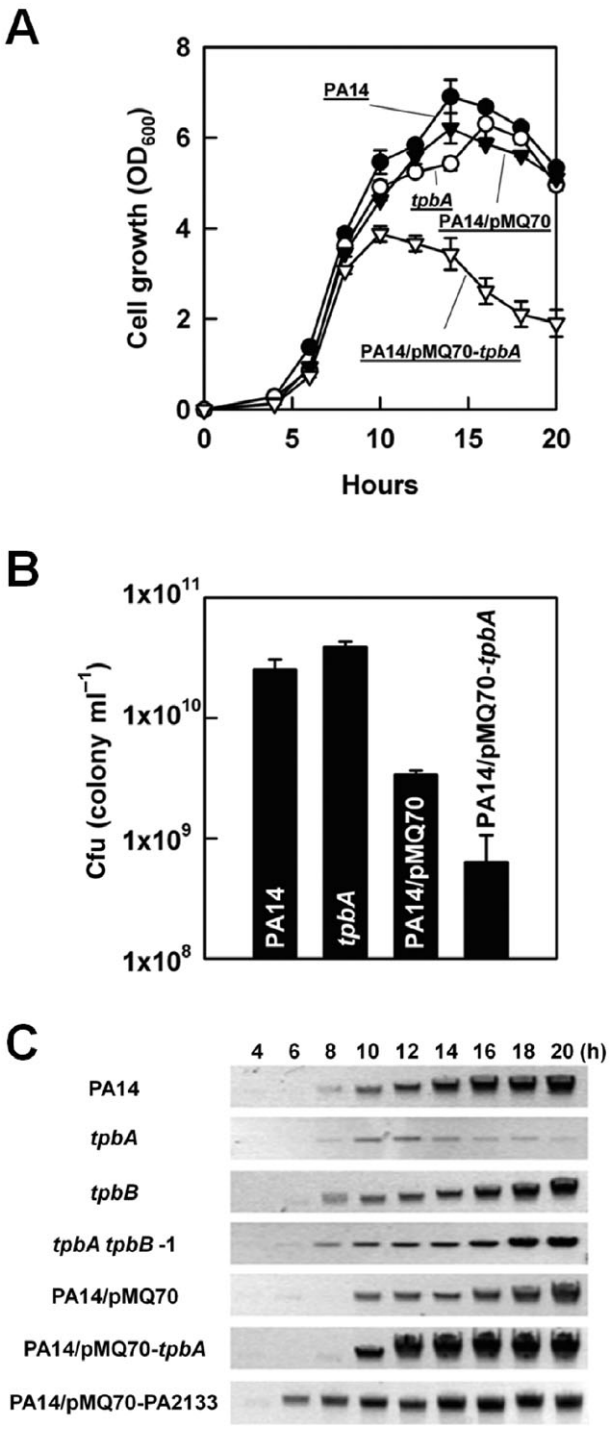

Fig. 3. TpbA controls cell viability/lysis.
A. Growth of PA14 wild-type and the *tpbA* mutant in LB and PA14/pMQ70 and PA14/pMQ70-*tpbA* in LB with 300 μg ml^{-1} carbenicillin at 37°C at 250 rpm. Because of the aggregative phenotype of the *tpbA* mutant upon dilution with fresh medium (Ueda and Wood, 2009), direct measurement of the turbidity at 600 nm is not accurate for cell growth and lysis. Hence, bacterial cultures were sonicated at 3 W for 10 s prior to measurement of turbidity using a Sonic Dismembrator (Model F60, Thermo Fisher Scientific, Waltham, MA). Colony-forming unit (cfu) measurements confirmed that this sonication level did not affect cell viability.
B. Colony-forming units after 14 h of growth in LB at 37°C. Bacteria were grown in 25 ml for 14 h at 250 rpm, and 500 μl of each culture was harvested by centrifugation at 13 000 *g* for 10 min. Cell pellets were resuspended in 500 μl of 0.85% NaCl, sonicated at 3 W for 10 s, and 10 μl of the serially diluted bacterial suspension was spotted on LB plates containing 100 μg ml^{-1} ampicillin for PA14 and the *tpbA* mutant, or 300 μg ml^{-1} carbenicillin for PA14/pMQ70 and PA14/pMQ70-*tpbA*. Colony-forming units were determined using the drop plate method (Herigstad *et al*., 2001). Data are the average of two independent cultures ± SD.
C. Time-course analysis of eDNA via DNA electrophoresis (1% agarose gel) after growth in LB at 37°C. Cultures (1 ml) were centrifuged at 13 000 *g* for 10 min at each time point, and 500 μl of the supernatant was mixed with 50 μl of 3 M sodium acetate and 500 μl of isopropanol to precipitate eDNA. Samples were treated with RNase A.

eDNA is similar to gDNA

The mechanism by which eDNA is produced is not clear; it may come from living or lysed bacterial cells. In eucaryotes, apoptosis triggers gDNA fragmentation rather than random degradation (Berke, 1994). In our experiments, obvious DNA degradation was not observed when extracts were analysed by agarose gel electrophoresis (data not shown). To examine the quality of eDNA, we randomly chose five loci to amplify DNA fragments by PCR. Using the primers for *tpbB* (PA1120), *kchA* (PA1207), *knaA* (PA5021), *rhlI* (PA3476) and *pvdS* (PA2426), DNA fragments were successfully amplified from both eDNA samples from the supernatant of bacterial cultures and from gDNA purified from bacterial cells (Fig. S2). This indicates that eDNA is probably close to intact gDNA and does not undergo DNA fragmentation or severe degradation during its excretion.

The tpbA *mutant forms flat and thick biofilms*

To examine how the *tpbA* mutation affects biofilm architecture, biofilm formation was tested using a flow cell chamber with continuous flow 10 ml h^{-1} of 5% LB medium for 72 h at 37°C. The biofilms formed by the *tpbA* mutant were sixfold smoother and flatter (roughness coefficient 1.7 ± 0.2 for PA14 versus 0.3 ± 0.2 for the *tpbA* mutant) and sixfold thicker (10 ± 10 μm for PA14 versus 57 ± 17 μm for the *tpbA* mutant) than those of PA14 (Fig. S3). In addition, biofilm biomass increased eightfold (6 ± 7 $\mu m^3\ \mu m^{-2}$ for PA14 versus 50 ± 22 $\mu m^3\ \mu m^{-2}$ for the *tpbA* mutant), and substratum coverage increased 30-fold (2 ± 1% for PA14 versus 60 ± 30% for the *tpbA* mutant).

In this report, we have found that TpbA and five other c-di-GMP-related proteins (PA0169, PA2133, PA4781, PA4959 and PA5487) influence eDNA in *P. aeruginosa* via modulation of cellular c-di-GMP and that eDNA is inversely proportional to c-di-GMP concentrations.

During biofilm dispersion, a release of metabolites was observed with a marked decrease of intracellular c-di-GMP concentrations (Schleheck *et al*., 2009), indicating that releases of cellular components, like eDNA, may be related to the intracellular concentration of c-di-GMP. In addition, both gain- and loss-of-function analysis showed that TpbA activity increases *P. aeruginosa* cell lysis (Fig. 3A and B).

Consistently, the *tpbA* mutant released 10-fold less eDNA in comparison with the PA14 wild-type strain (Fig. 1). Also, adding the *tpbB* mutation to the *tpbA* mutant restored eDNA to that of the wild-type strain (Figs 1 and 3C). Overexpression of phosphodiesterase PA2133, encoding the EAL domain for c-di-GMP degradation, increased eDNA (Fig. 1). In addition, inactivation of three active diguanylate cyclases (PA0169, PA4959, PA5487), which reduces cellular c-di-GMP concentrations, increased eDNA, and inactivation of the phosphodiesterase PA4781 decreased eDNA (Fig. 2). Taken together, these results show cellular c-di-GMP levels influence eDNA for *P. aeruginosa*.

We also investigated the biofilm architecture of PA14 and the *tpbA* mutant using flow cell chambers and found that the *tpbA* mutant formed flatter and thicker biofilms than PA14 (Fig. S3). This indicates that the greatly increased accumulation of c-di-GMP in the *tpbA* mutant results in undeveloped biofilm structures (Fig. S3), less accumulation of eDNA (Figs 1 and 3C), as well as hyperbiofilm formation in 96-well polystyrene plates (Ueda and Wood, 2009). The regulatory mechanism of eDNA release by c-di-GMP is proposed in Fig. 4.

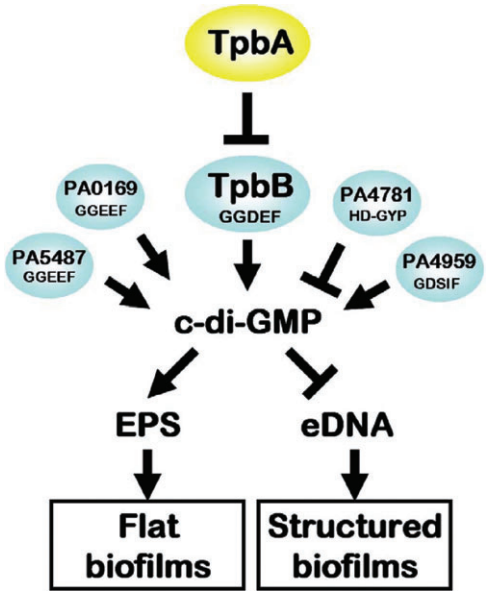

Fig. 4. Model for regulation of EPS/eDNA production by c-di-GMP. TpbA (tyrosine phosphatase) is a negative regulator of TpbB (a diguanylate cyclase), and TpbB produces c-di-GMP under the control of TpbA. In *P. aeruginosa*, most c-di-GMP is regulated by diguanylate cyclases PA0169, PA4959 and PA5487 that increase c-di-GMP thereby reducing eDNA. c-di-GMP is also regulated by a phosphodiesterase PA4781 that decreases c-di-GMP thereby increasing eDNA. Hence, cellular c-di-GMP increases EPS production, but decreases eDNA, and eDNA is necessary for the development of biofilm structure. Overproduction of EPS causes increased biofilm formation but these biofilms are flat so it may be necessary to reduce c-di-GMP levels by inducing TpbA in mature biofilms in order to release eDNA and complete biofilm maturation.

Cellular c-di-GMP has been found as a global regulator of diverse physiological processes, such as production of EPS, reduction of motility and production of virulence factors. *Pseudomonas aeruginosa* has divergent proteins for regulating cellular c-di-GMP levels including at least 41 GGDEF, EAL and HD-GYP domain proteins, which suggests that the concentration of cellular c-di-GMP is tightly regulated and governed by environmental cues. Because of this redundancy, it is not surprising that single mutations of GGDEF, EAL and HD-GYP do not cause large differences in eDNA. In contrast, the *tpbA* mutation and production of TpbA caused large decreases and increases, respectively, in eDNA (Figs 1 and 3C), and the *tpbA* mutation caused a large increase in c-di-GMP concentrations (Ueda and Wood, 2009).

One of the most striking phenotypes of the strains overproducing c-di-GMP is hyperbiofilm formation (Hickman *et al*., 2005; Kulasakara *et al*., 2006). Two of three HD-GYP proteins (PA2572, PA4108 and PA4781) degrade c-di-GMP in *P. aeruginosa* PAO1 because a mutation in PA4108 and PA4781 leads to fivefold greater c-di-GMP (Ryan *et al*., 2009). Both of these mutants formed flat biofilms while the wild-type strain formed mushroom-shaped biofilms (Ryan *et al*., 2009). Similarly, the *wspF* mutant, which accumulates more c-di-GMP, formed relatively flat and thicker biofilms (Hickman *et al*., 2005). These results show high concentrations of c-di-GMP cause the formation of flat biofilms with less structure, as we show for the *tpbA* mutant that formed flatter and thicker biofilms (Fig. S3). Since we found that the *tpbA* mutant has less eDNA and elevated cellular c-di-GMP, probably, TpbA decreases cellular c-di-GMP at the stage of biofilm maturation. Corroborating this result, we found *tpbA* is expressed primarily in the late stationary phase of planktonic cells (unpublished). Induction of TpbA in mature cells of a biofilm would then cause cell lysis which would provide the eDNA that has been shown necessary to develop the mushroom-shaped biofilm structure (Allesen-Holm *et al*., 2006). Therefore, TpbA appears important for controlling c-di-GMP levels and cell lysis as a means to regulate eDNA that is used for complex biofilm formation. Our results also imply that c-di-GMP levels are not fixed at high concentrations in biofilms and must decrease to facilitate mature biofilm architecture as well as dispersal.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Biofilm formation is positively controlled by c-di-GMP. Wild-type *P. aeruginosa* PA14 and the *tpbA* mutant were used to test biofilm formation using 96-well plates in LB at 37°C for 24 h with pMQ70 and pMQ70-PA2133. Overnight cultures of *P. aeruginosa* were diluted to a turbidity of 0.05 at 600 nm with fresh LB medium, and 150 μl of diluted bacterial culture was incubated in 96-well polystyrene plates. Biofilm formation was examined in 96-well polystyrene plates using crystal violet staining (Fletcher, 1977). To maintain the pMQ70 plasmid, carbenicillin (300 μg ml^{-1}) was added to the culture. Six wells were used for each culture. Data are the average of two independent cultures ± SD

Fig. S2. Comparison of PCR products with eDNA and gDNA. PCR was used to amplify five loci (*tpbB, kchA, knaA, rhlI* and *pvdS*) (Table S2) of the *P. aeruginosa* genome to compare eDNA (e) and gDNA (g). Experiments were performed with 50 ng of eDNA and gDNA from both wild-type PA14 and the *tpbA* mutant. The PCR conditions were denaturation at 95°C for 5 min, 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min. PCR products were analysed by 1% agarose gel electrophoresis.

Fig. S3. Biofilm structure in flow cells for PA14 and the *tpbA* mutant. *Pseudomonas aeruginosa* PA14 and the *tpbA* mutant formed biofilms in flow cell chambers in 5% LB at 37°C. After 72 h of incubation, biofilms were stained with SYTO9 for 20 min in the dark. Biofilm images were scanned using a confocal microscope. The experiment was repeated and images were randomly scanned from six different positions on the glass slides. Images was produced by IMARIS, and representative images are shown. Biofilm characterization (biomass, substratum coverage, thickness and roughness coefficient) was determined by COMSTAT.

Table S1. Strains used in this study.

Table S2. Primers used in this study.

Table S3. Estimation of cell lysis for PA14 and the *tpbA* mutant by (i) the total protein assay and (ii) live/dead staining.

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