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Tyrosine phosphatase TpbA controls rugose colony formation in *Pseudomonas aeruginosa* by dephosphorylating diguanylate cyclase TpbB

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ABSTRACT

Tyrosine phosphatase TpbA in *Pseudomonas aeruginosa* PA14 is a negative regulator of the diguanylate cyclase TpbB. Inactivation of TpbA caused rugose colony morphology which is related to cell persistence in clinical infections. We show here that TpbA is a dual specific tyrosine phosphatase, that TpbB is phosphorylated, and that TpbA controls phosphorylation of TpbB at both Tyr and Ser/Thr residues *in vivo* as detected by Western blot analysis. In addition, TpbB is demonstrated to be a substrate of TpbA *in vitro* using purified enzymes. Thus, TpbA controls the rugose morphology in *P. aeruginosa* by dephosphorylating TpbB.

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is responsible for many biofilm infections including those associated with ventilator-associated pneumonia, urinary and peritoneal dialysis catheters, bacterial keratitis, otitis externa, burns, and lungs [1]. Persistence of this bacterium is linked to its ability to form biofilms [2] and rugose small-colony variants (RSCVs) [3] which are characterized by wrinkled, small colonies and an elevated capacity to form biofilms.

In *P. aeruginosa* PA14, we found previously that *tpbA* (PA3885) encodes a conserved tyrosine phosphatase (*t*yrosine *p*hosphatase related to *b*iofilm formation) that when inactivated, converts the smooth wild-type strain into an RSCV, with dramatically increased biofilm formation, attachment, and aggregation along with decreased swimming and no swarming [4]. The mechanism of the RSCV formation in the *tpbA* mutant is linked to increased 3,5-cyclic diguanylic acid (c-di-GMP) that results in elevated activity of the *pel* polysaccharide locus [4]; this result was the first link between bacterial tyrosine phosphatase activity and c-di-GMP formation. Furthermore, TpbA increases extracellular DNA production by decreasing c-di-GMP concentrations [5]. c-di-GMP is an ubiquitous intracellular second messenger that acts as a central regulator in bacterial physiology, especially in regulating the transition between motile and sessile states [6]. c-di-GMP is synthesized from two molecules of guanosine-5′-triphosphate (GTP) by diguanylate cyclases (DGCs) that contain GGDEF domains, and degraded by phosphodiesterases (PDEs) that contain EAL or HD-GYP domains [6]. PA14 has 37 putative c-di-GMP related proteins, including 16 proteins with a DGC domain, 5 with a PDE domain, and 16 that contain both domains [7]. Critically, genetic screening indicated that inactivation of *tpbB* (PA1120, *yfiN*), which encodes an active DGC [7], suppressed the phenotype observed in the *tpbA* mutant [4]. This implies that TpbA regulates c-di-GMP concentrations through TpbB. Our original results for the role of TpbB in RSCV morphology formation were recently verified by an independent group that corroborated that TpbB is important for persistence related to cystic fibrosis [8]. However, how TpbA regulates TpbB has not been elucidated yet.

In this study, we demonstrate that TpbA dephosphorylates TpbB *in vivo*. In addition, we show that TpbB serves as the substrate of TpbA *in vitro*, providing direct evidence of TpbA/TpbB interactions.

2. Materials and methods

2.1. Cell culture

Strains used in this study are listed in Table 1. *P. aeruginosa* PA14 (wild-type) and its isogenic mutants were obtained from the Harvard Medical School [9]. *P. aeruginosa* and *Escherichia coli* were routinely grown in Luria–Bertani (LB) medium [10] at 37 °C unless noted. Gentamicin (15 μg/mL) was used for growth of the *P. aeruginosa tpbA* transposon mutant, carbenicillin (300 μg/mL) was used to maintain pMQ70-*tpbB*, and kanamycin (50 μg/mL) was used to maintain plasmid pET28b-*tpbA*.

2.2. Protein purification

Recombinant TpbA with a 6X-His-tag at the carboxy terminus (TpbA-cHis) was produced in *E. coli* BL21 (DE3) with plasmid

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Table 1
Strains and plasmids used in this study. Gm^R, Km^R, Car^R, and Ap^R indicate gentamicin, kanamycin, carbenicillin, and ampicillin resistance, respectively.

Strains and plasmids	Relevant genotype	Source
P. aeruginosa		
PA14	Wild-type strain	[9]
tpbA	PA14_13660 Ω Mar2xT7, Gm^R	[9]
E. coli		
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm* λ(DE3) Ω *plac*UV5:: T7 polymerase	Novagen
Plasmid		
pMQ70-*tpbB*	P_{BAD}::*tpbB*, complementation plasmid, Car^R, Ap^R	[4]
pET28b-*tpbA*	P_{T7}::*tpbA-cHis*$^+$, expression vector for TpbA-cHis, Km^R	[4]

pET28b-*tpbA* by inducing with 1 mM IPTG and was purified using Ni–NTA resin as described previously [4]. The purified TpbA-cHis was dialyzed against buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 10% glycerol) at 4 °C overnight and concentrated using a 10 kDa cut-off centrifugal filter unit (Millipore, Billerica, MA).

To purify TpbB, *tpbA* was transformed with pMQ70-*tpbB* [4] and used to produce the full-length TpbB with a 6X-His-tag at the carboxy terminus (TpbB-cHis). After 36 h of incubation from a single colony, 1 mL was used to inoculate 1 L of LB medium supplemented with 300 μg/mL carbenicillin and 0.05% arabinose; this culture was incubated at 37 °C with shaking. Early stationary-phase cells were harvested by centrifugation at 8000*g* for 10 min at 4 °C. Cells were re-suspended in 20 mL lysis buffer (50 mM Tris–HCl, pH 8.0) with phosSTOP phosphatase inhibitor cocktail (Roche, Indianapolis, IN) and 100 μl protease inhibitor cocktail (Sigma, St. Louis, MO). Cells were disrupted twice by a French Press (Thermo Electron Corporation, Waltham, MA). Non-soluble cellular debris was removed by centrifuging twice at 15,000*g* for 30 min. The whole cell lysate was centrifuged at 100,000*g* for 1 h at 4 °C to separate the soluble fraction from the total membrane fraction [11]. The membrane fraction was re-suspended in 4 mL purification buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10% glycerol, 1.5% TX-100 with Roche phosSTOP phosphatase inhibitor cocktail and 10 μl Sigma protease inhibitor cocktail) and incubated on ice overnight. TpbB-cHis was then purified using Ni–NTA agarose resin (Qiagen, Valencia, CA) as described by the manufacturer's protocol. The protein was eluted with purification buffer supplemented with 100 mM imidazole. Protein concentrations were assayed by the BCA assay (Pierce, Rockford, IL).

2.3. Western blot

Two sets of Western blot experiments were performed. The first was to detect phosphorylation of TpbB *in vivo*. In this experiment, whole membrane TpbB proteins from PA14/pMQ70-*tpbB* and *tpbA*/pMQ70-*tpbB* were isolated from early stationary-phase cultures as described in the protein purification section. Then the membrane protein was re-suspended in lysis buffer containing 1.5% TX-100, and the protein concentration was assayed by the BCA assay. The same amount of membrane protein (2 μg) was loaded into each well of a 10% SDS–PAGE gel, then transferred to a PVDF membrane, which was then blocked with 4% BSA in TBST (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The blot was incubated with a 1:1000 dilution of anti-phosphotyrosine antibody 4G10 (Millipore) or anti-phosphoserine/threonine antibody (BD Science, Franklin Lakes, NJ) at 4 °C for overnight. After washing three times with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Cell signaling Technology, Danvers, MA) at room temperature for 1 h and detected by Super Signal Pico substrate (Pierce) after washing with TBST. Then the blot was stripped to remove the antibodies, blocked again, and re-probed with 1:2000 dilution of His-tag antibody (Cell Signaling Technology) using the same procedure. The other Western blot analysis was performed to detect phosphorylation of purified TpbB samples. In this experiment, 300 ng of purified TpbB were loaded into each well of a SDS–PAGE gel instead of using the whole membrane fraction.

2.4. TpbA phosphatase assay

To examine whether TpbA is a dual specific tyrosine phosphatase, TpbA activity was checked using the tyrosine phosphopeptide END[pY]INASL (Promega, Madison, WI) and the threonine phosphopeptide KR[pT]IRR (Millipore) using the Tyrosine Phosphatase Assay System (Promega). TpbA (10 μg) was incubated with 50 μM of peptide in 50 μl of reaction buffer (10 mM Tris–acetate, pH 5.5, 10 mM $MgCl_2$) for 2 h at 37 °C. The reaction was quenched by adding 50 μl of a molybdate dye solution and incubating at room temperature for 30 min. Released phosphate was quantified by measuring the absorbance at 630 nm.

To check whether TpbB is the substrate of TpbA, the buffer for purified TpbB was changed to 50 mM Tris–acetate, pH 5.5 with 0.1% TX-100 using a 30 kDa centrifuge filter unit. Then equal amounts of TpbA and TpbB were incubated at 37 °C in reaction buffer for 2 h. The reaction was quenched by adding SDS-loading dye and heating at 95 °C for 10 min. For the control, TpbB (3 μg) was treated using the same procedure but without adding TpbA.

2.5. c-di-GMP phosphodiesterase assay

To check whether TpbA is a phosphodiesterase which degrades c-di-GMP, PDE activity was assayed as previously described [12] using high-performance liquid chromatography (HPLC) to quantify c-di-GMP concentrations. Purified TpbA (3 μg) was incubated with 0.1 mM c-di-GMP (BIOLOG Life Science Institute, Bremen, Germany) in 50 μl of reaction buffer (50 mM Tris–HCl, pH 9.0 or 50 mM Tris–acetate, pH 5.5, with 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT, and 5 mM $MgCl_2$) at 37 °C for 1 h, and the reaction was quenched by adding 10 mM $CaCl_2$ followed by heating at 98 °C for 10 min. The reaction products were analyzed by HPLC using a C18 reverse-phase column (150 × 3.9 mm, 4 μm, Nova-Pak, Waters), and nucleotides were detected at a wavelength of 254 nm. Phosphodiesterase YahA from *E. coli* was used as a positive control and used to generate 5′-phosphoguanylyl-(3′ → 5′)-guanosine [12] from c-di-GMP. Guanosine monophosphate was obtained from Sigma.

2.6. TpbB diguanylate cyclase assay

TpbB diguanylate cyclase activity was assayed by ^{31}P NMR *in vitro*. The reaction was initiated by the addition of 10 μg of TpbB into a 400 μl reaction mixture (5 mM GTP in 50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 10 mM TX-100, and 5 mM DTT containing 30% D_2O) in the NMR tube. ^{31}P NMR spectra were recorded using a Varian INOVA 400 spectrometer. The spectra were accumulated with proton decoupling, 90° pulses, an acquisition time of 1.6 s, first delay of 1.0 s, and with the temperature maintained at 37 °C throughout the experiment. Phosphoric acid (85%) was used as an external standard for the chemical shift at 0 ppm. The reaction mixture was kept in the probe of the instrument during the experiment, while spectra were recorded at various time intervals. As a negative control, the same amount of TpbB was denatured by heating at 95 °C for 10 min before adding to the reaction mixture. The product c-di-GMP concentration was calculated as the percentage of the integrated intensity of the substrate peak by comparing the sum of the integrated intensities of the substrate and product peaks in each spectrum.