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Oxygen promotes biofilm formation of *Shewanella putrefaciens* CN32 through a diguanylate cyclase and an adhesin

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Although oxygen has been reported to regulate biofilm formation by several *Shewanella* species, the exact regulatory mechanism mostly remains unclear. Here, we identify a direct oxygen-sensing diguanylate cyclase (DosD) and reveal its regulatory role in biofilm formation by *Shewanella putrefaciens* CN32 under aerobic conditions. *In vitro* and *in vivo* analyses revealed that the activity of DosD culminates to synthesis of cyclic diguanylate (c-di-GMP) in the presence of oxygen. DosD regulates the transcription of *bpfA* operon which encodes seven proteins including a large repetitive adhesin BpfA and its cognate type I secretion system (TISS). Regulation of DosD in aerobic biofilms is heavily dependent on an adhesin BpfA and the TISS. This study offers an insight into the molecular mechanism of oxygen-stimulated biofilm formation by *S. putrefaciens* CN32.

Shewanella species are widely distributed in diverse environments due to their flexible respiration. These facultative anaerobic bacteria can use a wide range of electron acceptors and are classified in the group of dissimilatory metal-reducing bacteria[1]. Their respiratory versatility allows them to play an important role in the geochemical cycling of iron, manganese and carbon sources, as well as in bioremediation and bio-engineering applications[2,3]. As such, *Shewanella* species have been increasingly recognized as the model system for environmental bacterial metabolism.

Biofilm is a common lifestyle for many bacteria[4]. Microbial cells are embedded in a matrix of self-synthesized extracellular polymeric substances which provides multiple advantages for the survival of the microbial community[5]. *Shewanella* species can form biofilms under diverse environments. For instance, *Shewanella* strains have been isolated from marine biofilms and the surface of green algae[6,7]. The formation of microcolonies on the surface of iron oxides also suggests that *Shewanella* species might tend to attach and establish stable contact on mineral surfaces[8]. In bioelectrochemical systems, *Shewanella* cells form biofilms on electrode surfaces, which is important for transferring electrons from cells to electrodes[9,10].

The biofilm formation by *Shewanella* is affected by numerous factors, amongst which, oxygen might play a critical role. As a ubiquitous oxidant in nature, oxygen can significantly affect the pH, redox state and reduction potential in many abiotic and biotic systems. It is worth noting that *Shewanella* species thrive in oxic-anoxic interfaces, where redox conditions change rapidly with frequent shifts in the main electron acceptors[11]. It has been demonstrated that oxygen can affect the formation and three-dimensional structure of a community formed by *Shewanella* species, such as pellicles at air-liquid interfaces, aggregates in aerobic chemostat cultures and biofilms in hydrodynamic flow cells[12–14]. Biofilms formed under aerobic conditions show a hollow and seeding dispersal structure, while a round and densely-packed structure under anaerobic conditions[15]. The rapid detachment of cells from established aerobic biofilms of *Shewanella* has been observed in flow cell chambers after the flow is halted. Oxygen depletion is assumed to be the trigger of detachment, suggesting the quick detection and response of the biofilm to oxygen fluctuations[14]. However, the underlying oxygen sensing and responding mechanisms are mostly unknown[13].

This study aims to explore the mechanism of oxygen sensing and the molecular response in *Shewanella* biofilms. Here, in *S. putrefaciens* CN32, we identified the direct oxygen-sensing diguanylate cyclase (DosD)

which regulates the expression of the RTX adhesin and corresponding type I secretion system (TISS) that consequently affect biofilm formation.

Results

DosD enhances aerobic biofilm formation by *S. putrefaciens* CN32. Genome annotation predicts that Sputcn32_3244 in *S. putrefaciens* CN32 encodes a putative diguanylate cyclase (DGC), composed of an N-terminal globin domain and a C-terminal GGDEF domain (Figure 1A). Given the typical function of the globin domain as a gas sensor[16], we therefore postulated that Sputcn32_3244 might encode an oxygen sensing DGC (*dosD*) in *S. putrefaciens* CN32.

To elucidate the function of *dosD*, a mutant with an in-frame deletion of the *dosD* (Δ*dosD*) was constructed and analyzed. Growth and biofilm formation between wild-type (WT) and Δ*dosD* strains were compared under both aerobic and anaerobic conditions. The biofilm that formed on the wall of glass tubes in different growth phases was quantified using the crystal violet staining method. The biofilm mass formed by the Δ*dosD* was significantly less than that of WT after entering the late-exponential phase of aerobic growth, but growth rates were not substantially different (Figure 1B). By contrast, no significant difference in either biofilm formation or growth rate was observed between the two strains under anaerobic conditions (Supplementary Information, SI, Figure S1). The *dosD* is localized downstream of the Sptcn32_3243 gene with the same transcriptional direction and a 54-bp intergenic region. To assess the transcriptional unit of *dosD*, reverse transcription PCR analysis was performed using primer pairs targeting a possible contiguous mRNA between Sputcn32_3243 and *dosD*. No PCR product was detected, indicating independent transcription of *dosD*. The sequence of *dosD* with its promoter region was cloned to construct a complementary plasmid, pBGdosD. The biofilm formed by the Δ*dosD* containing pBGdosD was restored to a level similar to WT (Figure 1C).

The surface attachment and motility, which play opposite roles in biofilm formation, were assessed for the WT and the Δ*dosD*. Attachment is important in the initial stage of biofilm formation. Results revealed that the attachment ability of Δ*dosD* cell on coverslips was significantly reduced compared to WT cells (Figure 1D). Motility assay, however, showed no substantial distinction between the WT and Δ*dosD* strains in the time tested (SI, Figure S2). Thus, DosD is likely to affect cellular activity relating to attachment.

Oxygen stimulates the diguanylate cyclase activity of DosD. To examine whether *dosD* encodes an active DGC, His-tagged DosD was expressed in *E. coli* BL21(DE3) and purified. Enzymatic analysis of purified DosD was subsequently performed under both oxygen-binding (oxy-DosD) and oxygen-deprivation (deoxy-DosD) conditions. Oxy-DosD showed very high activity of DGC compared to deoxy-DosD (Figure 2A; SI, Figure S3). At the initial stage of enzymatic reaction, one mole of oxy-DosD converted 1.68 ± 0.04 mole of GTP into cyclic di-GMP, while deoxy-DosD converted only 0.52 ± 0.02 mole of GTP into cyclic di-GMP. Absorption spectroscopy analysis of the recombinant DosD showed that depriving oxygen from DosD resulted in a shift of the peak from 411 nm to 426 nm, implying the transition from the oxygen-binding Fe(II) state to the unligated Fe(II) state (SI, Figure S4).

Intracellular activity of DosD was further examined. The intracellular levels of c-di-GMP in the WT and the Δ*dosD* were compared when grown under aerobic and anaerobic conditions, respectively. Under aerobic growth, Δ*dosD* showed a significant decrease (~40%)

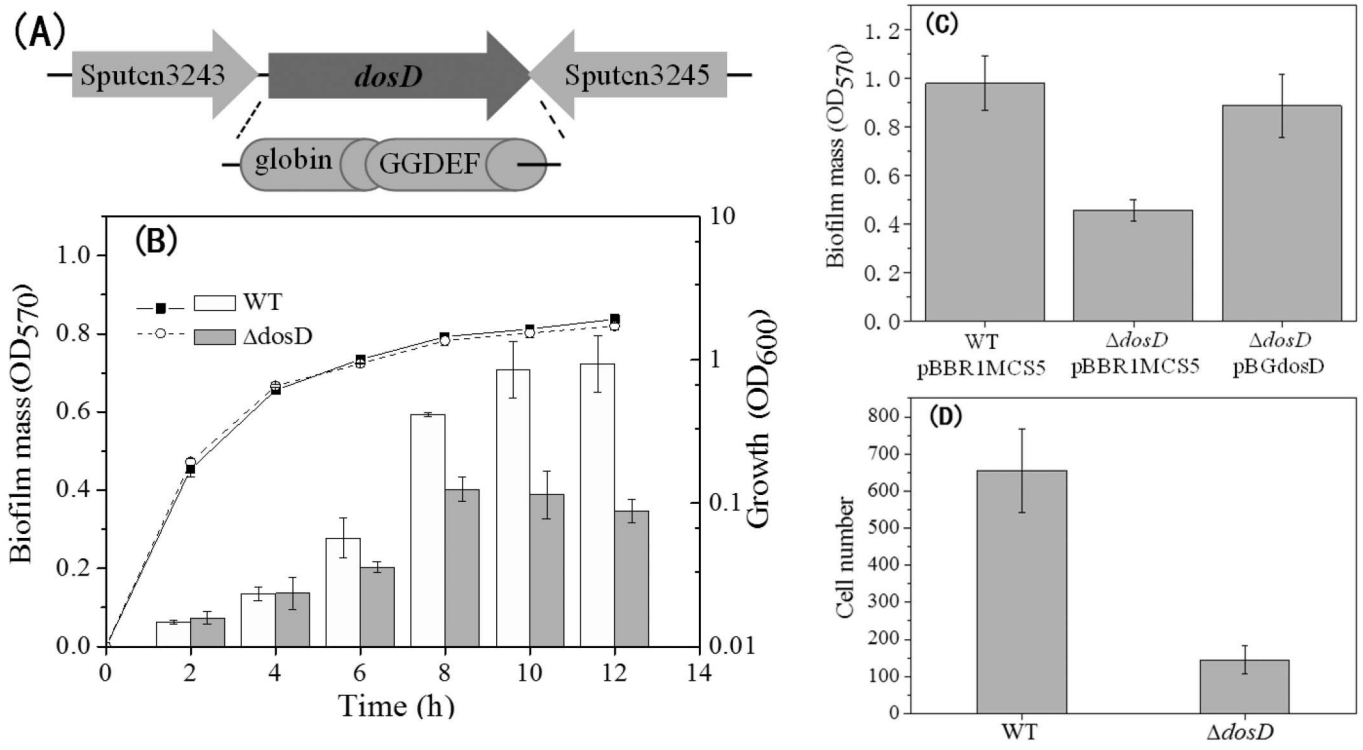

Figure 1 | DosD positively regulates aerobic biofilm production in *S. putrefaciens* CN32. (A) Schematic of the genetic arrangement of DosD and its domain architecture. (B) Aerobic biofilm mass profiles of WT (white columns) and Δ*dosD* (gray columns) with simultaneous determination of growth of WT (solid line) and Δ*dosD* (dashed line). (C) Biofilm formation by Δ*dosD* complemented by vector-bearing *dosD* driven by its native promoter. The empty vector was transformed into WT and Δ*dosD* mutants as a control. (D) Attachment abilities of WT and Δ*dosD*. The average number of cells attached to the cover slips was counted in randomly picked areas of ten microscopic images. Each experiment was repeated at least three times and representative results are provided. Error bars refer to the standard deviation of the three samples.

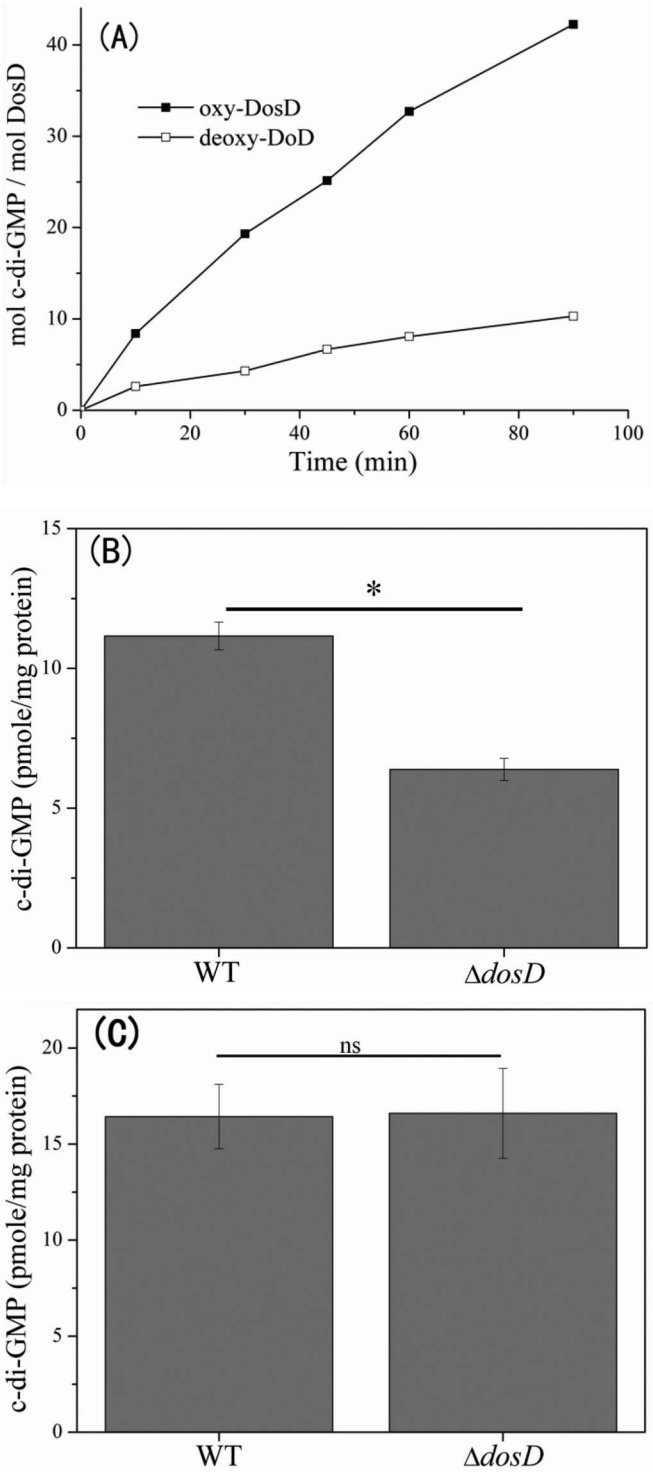

Figure 2 | DosD is a diguanylate cyclase activated by oxygen. (A) Enzymatic activity of DosD with and without oxygen binding was assayed for its ability to catalyze the conversion of GTP to c-di-GMP. Intracellular levels of c-di-GMP in WT and Δ*dosD* under aerobic growth (B) and anaerobic growth (C). Error bar indicates standard deviation of the averages of five independent samples. Asterisk indicates the statistical significance of the difference and sn indicates no significant difference ($P < 0.05$).

in intracellular concentration of c-di-GMP compared with the WT. Under anaerobic growth, no significant difference in c-di-GMP concentration was detected for the two strains (Figure 2B&C; SI, Figure S5). In addition, the expression of DosD in *E.coli* BL21(DE3) caused

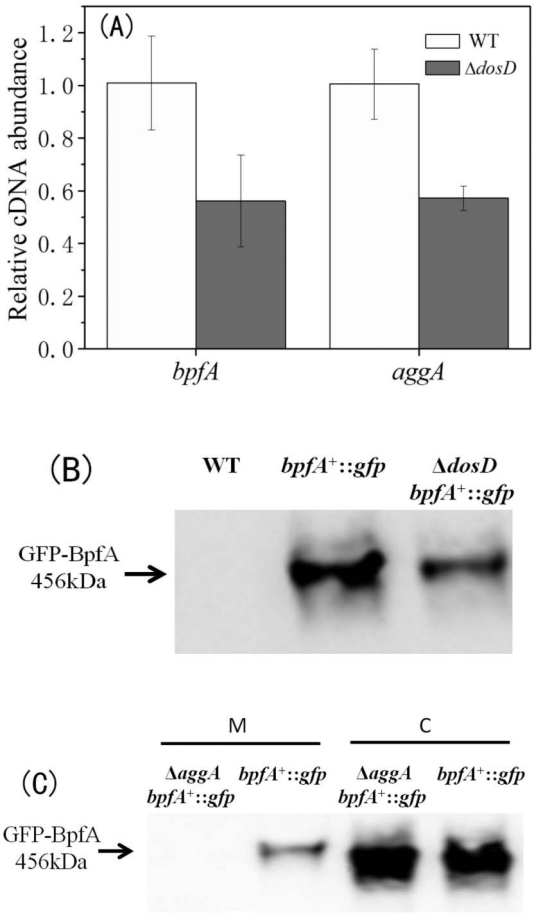

Figure 3 | DosD regulates the transcription and secretion of *bpfA*. (A) The transcription levels of *bpfA* and *aggA* in Δ*dosD* relative to WT. The quantity of cDNA was normalized to the abundance of 16S cDNA in the qRT-PCR analysis. (B) The level of BpfA in Δ*dosD bpfA*$^+$*::gfp* and *bpfA*$^+$*::gfp*. WT was set as a control. (C) Localization and abundance of BpfA in Δ*aggA bpfA*$^+$*::gfp* and *bpfA*$^+$*::gfp*. M indicates the sample of membrane fractions and C indicates the sample of cytoplasm fraction.

obviously reddish and rugose morphology of clones when grown on Congo-Red plates, suggesting that DosD in *E.coli* was also active and enhanced the production of extracellular polymeric substances (SI, Figure S6). These results from *in vitro* and *in vivo* analyses verify that DosD is a DGC and its activity is stimulated by oxygen.

DosD regulates the expression of an adhesin operon. To explore the downstream targets of DosD involved in biofilm formation by *S. putrefaciens* CN32, the transcription of putative genes encoding surface appendages, proteins and enzymes for synthesizing polysaccharides which might contribute to biofilm formation was analyzed (SI, Table S1). The quantitative RT-PCR (qRT-PCR) results showed no significant transcriptional alterations, except for the *bpfA* encoded by Sputcn32_3591. The transcription of *bpfA* decreased by ~50% in the Δ*dosD* compared with the WT (Figure 3A).

Differential *bpfA* mRNA levels detected in the WT and Δ*dosD* were further evaluated at the protein level. To detect BpfA in western blotting, green fluorescent protein (*gfp*) was translationally fused to *bpfA* in the genome to construct derivatives with GFP-labelled BpfA (*bpfA*$^+$*::gfp*). Since the signal peptides for secretion of RTX adhesin proteins are located in the C-terminus[17], GFP was fused into the N-terminal domain of BpfA. Western blotting analysis was carried out to detect GFP-fused BpfA in the *bpfA*$^+$*::gfp* and the Δ*dosD bpfA*$^+$*::gfp*. The bands corresponding to GFP-fused BpfA could be specifically

detected in the samples of GFP-labeled strains but not in the unlabelled-WT. The BpfA level in the Δ*dosD* was obviously reduced than in the WT (Figure 3B).

The BpfA belongs to the subfamily of large repetitive RTX adhesins, and is predicted to start a seven-gene operon including a type I secretion system (TISS) apparatus[18,19]. In this study, reverse transcription PCR targeting for intergenic regions confirmed that the *bpfA* operon consisted of seven putative genes, encoding a cell surface adhesin (*bpfA*), three components of the TISS (Sputcn32_3592-3594), an OmpA-like family protein (Sputcn32_3595), a cysteine protease (Sputcn32_3596), and a GGDEF/EAL domain protein (Sputcn32_3597) (SI, Figure S7). The AggA encoded by Suptcn32_3594 is one component of the TISS and has been shown to be involved in the formation of pellicles, biofilms and hyperaggregates[12,20,21]. Thus, transcription of *aggA* was also analyzed in this study. Similar to *bpfA*, the mRNA level of *aggA* also decreased in Δ*dosD* compared to WT (Figure 3A).

AggA is presumably involved in the transportation of BpfA to cell surface, thus BpfA levels in membrane and cytoplasm fractions of the Δ*aggA* were compared with those of the WT. No substantial difference of BpfA in cytoplasm fractions between the Δ*aggA bpfA*$^+$::*gfp* and the *bpfA*$^+$::*gfp* was detected. However, BpfA was not detected in the membrane fraction of Δ*aggA bpfA*$^+$::*gfp* (Figure 3C), indicating that AggA has no effect on the expression of BpfA, but is essential for its cell surface location.

c-di-GMP positively regulates biofilms and the expression of *bpfA*. To examine the role of c-di-GMP in the biofilm phenotype of *S. putrefaciens* CN32, a DGC AdrA was cloned from *Salmonella typhimurium* 14028 and constitutively expressed in the Δ*dosD* driven by *lac* derived $P_{A1/04/03}$ promoter[22]. Δ*dosD* constitutively expressing AdrA from $P_{A1/04/03}$ demonstrated a significantly increased biofilm compared with no expression of AdrA, although the biofilm was not fully restored to the level of the WT (Figure 4A). In addition, the transcription of *bpfA* in the Δ*dosD* expressing AdrA was also significantly increased compared with the Δ*dosD* without AdrA expression (Figure 4B).

DosD promotes biofilm formation dependent on the TISS. The role of BpfA and AggA in DosD-regulated aerobic biofilm formation was subsequently evaluated. In this case, *bpfA* and *aggA* were deleted in the WT and the Δ*dosD*, respectively. BpfA in *S. putrefaciens* CN32 is a large protein with a molecular weight of 429 KDa with repetitive sequences. To thoroughly deactivate *bpfA*, primers were designed to introduce a frameshift mutation which produced ~250 stop codons in the remaining sequence of the *bpfA* in genome. The biofilm analysis shows that Δ*bpfA*, Δ*dosD*Δ*bpfA*, Δ*aggA* and Δ*dosD*Δ*aggA* had similar and greatly impaired biofilm formation compared with WT (Figure 5). This result suggests that BpfA is the primary contributor to DosD-regulated biofilm formation. AggA might not directly contribute to DosD-regulated biofilm formation, but rather act indirectly by affecting BpfA secretion.

Discussion

Bacteria utilize diverse strategies to sense physiologically important gases, like oxygen, and subsequently orchestrate global cellular response. One such important response is the regulation of biofilm development. The biofilm structure of *S. oneidensis* MR-1 varies remarkably with oxygen availability[15]. The rapid detection and response of *Shewanella* biofilm to oxygen fluctuations has been previously reported[15]. Cultures of *Shewanella* cells under aerobic chemostat conditions tend to autoaggregate, which has a similar physiology and morphology to biofilm[15]. For *Shewanella* species, oxygen-stimulated biofilm development may help alleviate oxidative stress and/or facilitate the development of hypoxic and even

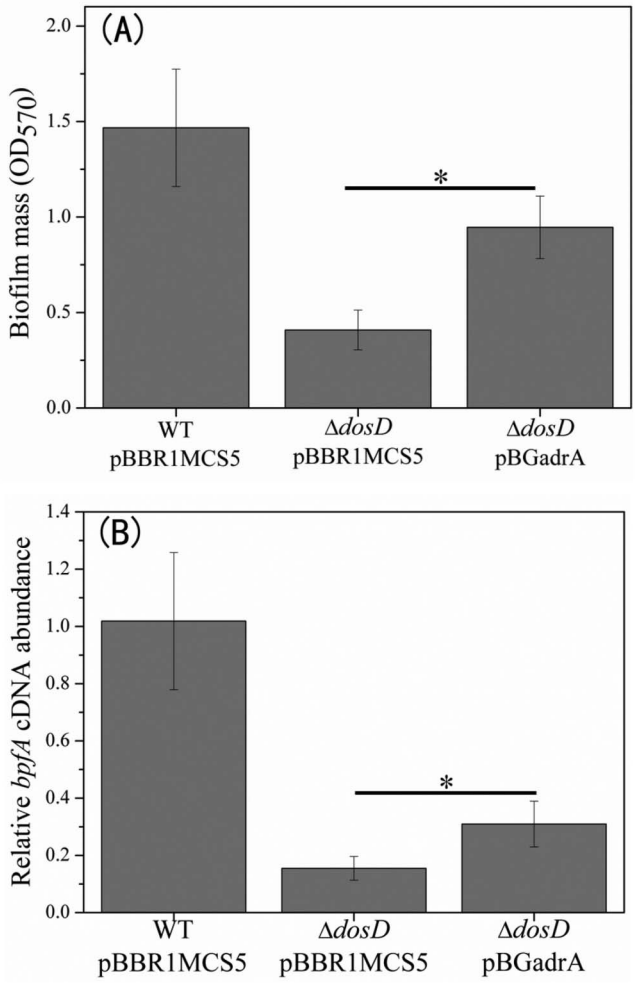

Figure 4 | Expression of AdrA increased biofilm (A) and *bpfA* transcription (B) in Δ*dosD*. Error bar indicates the standard deviation of the averages of three independent samples. Asterisk indicates the statistical significance of the difference ($P < 0.05$).

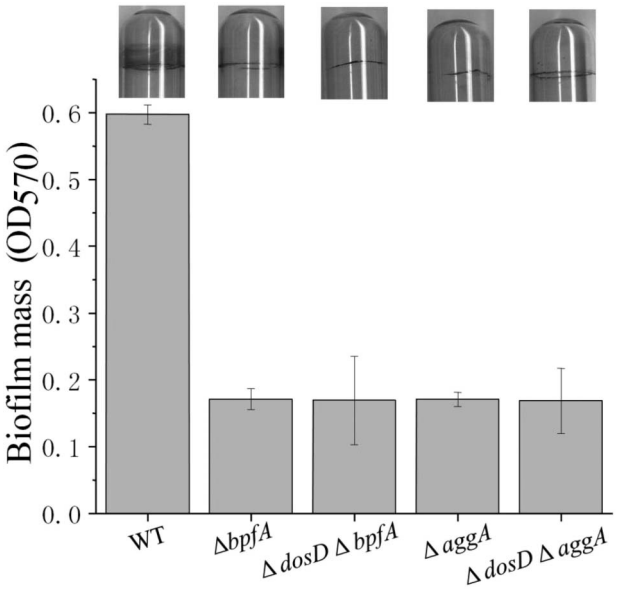

Figure 5 | The *bpfA* operon is required for DosD-induced biofilm enhancement. Biofilms formed by Δ*bpfA*, Δ*dosDbpfA*, Δ*aggA* and Δ*dosDaggA* after 12 h of growth. Representative glass tubes after stained by crystal violet for biofilm assay are shown on the top.

anaerobic conditions within the biofilm. However, the detailed mechanisms for oxygen sensing and the consequent regulation of biofilm development are not well defined.

This study aims to elucidate the molecular mechanism by which *Shewanella* species respond to the oxygen fluctuation and regulate their biofilm formation. Bioinformatic analysis shows that some of *Shewanella* species including *S. putrefaciens* CN32 encode a DGC protein with a globin domain[23]. Globin domain proteins are conserved in the three kingdoms of life[24]. In bacteria, a class of globin-coupled sensors (GCSs) has been identified. GCSs are liable to bind gaseous ligands and are assumed to transmit a signal of conformational change as a response to fluctuation of gas levels to the catalytic domain. A putative GCS in *S. putrefaciens* CN32, designated here as DosD, is a protein with an N-terminal globin domain and a C-terminal GGDEF domain. The amino acid sequence alignment of the N-terminal globin domain in DosD shows similarity with some GCSs known to function as oxygen-sensing DGCs (SI, Figure S7)[25,26]. It is possible that globin-coupled DGCs might participate in the response of this bacterium to oxygen in various environments by regulating the c-di-GMP level in the cell.

In this study, we demonstrated that DosD is responsible for ~40% of the c-di-GMP synthesis under aerobic growth, but has no effect under anaerobic growth, indicating that DosD could modulate the aerobic c-di-GMP pool. Enzymatic characterization of the recombinant DosD offers direct evidence for its activity as an oxygen-activated DGC. c-di-GMP is an important secondary messenger regulating diverse physiological processes in a wide variety of bacteria[27]. It is synthesized by DGCs and hydrolyzed by phosphodiesterases (PDEs), which have normally been identified by the presence of catalytic domains with the conserved amino acid sequences GGDEF and EAL/HD-GYP, respectively[28]. The level of c-di-GMP is modulated by a series of DGCs and PDEs. The activities of these DGCs and PDEs are affected by various environmental cues and in turn regulate downstream targets[29]. *Shewanella* species encode a large number of putative c-di-GMP-related proteins than many other bacterial species. Taking *S. putrefaciens* CN32 as an example, its genome presumably encodes 43 GGDEF domain proteins, 25 EAL domain protein and 21 proteins possessing both domains. Encoding for so many c-di-GMP-related proteins suggests that c-di-GMP plays important and subtle roles in the lifecycles of *Shewanella* species. Nevertheless, information about the role of c-di-GMP in *Shewanella* species is very limited so far, and less is known about the detailed signal transduction and regulatory pathway. One of the important functions of c-di-GMP is to control the shifts between the planktonic and sessile growth modes of bacteria[30]. We demonstrated the regulation of c-di-GMP in biofilm formation through hetero-expressing a DGC, AdrA, in *S. putrefaciens* CN32. AdrA is an active DGC responsible for ~60% of the c-di-GMP synthesized in *Salmonella* Typhimurium[31] and has been used to investigate the role of c-di-GMP in other bacteria[31,32]. In the present study, AdrA was expressed in the Δ*dosD* and partially restored the defected biofilm cause by the *dosD* deletion to the level of the WT, suggesting a possibly higher activity of DosD than AdrA in *S. putrefaciens* CN32.

The role of c-di-GMP in biofilm formation by *Shewanella* species has been explored in several studies. A degenerated GGDEF protein MxdA was found to indirectly modulate the c-di-GMP level and affect biofilm formation by *S. oneidensis*[33]. Nitric oxide can regulate levels of c-di-GMP in *S. oneidensis* and *woodyi*[34,35]. However, few works reveal the c-di-GMP regulatory pathway in *Shewanella* species from signal sensing to target regulation. In this work, the downstream target of DosD was identified as the *bpfA* operon mainly encoding for a RTX adhesin and its secretion system, TISS. RTX proteins are Ca^{2+}-responsive, high molecular weight, multidomain, loosely-associated cell surface proteins. This protein family is known to promote bacteria-bacteria or bacteria-host attachment[19]. BpfA has been identified as an important component of biofilm formation through random transposon insertion mutagenesis in *S. oneidensis*[18]. Transcriptional analysis in this work shows that *bpfA* initiates an operon (Sputcn32_3591-3597) including a TISS, an ompA-like protein, a hypothetical protein, and a GGDEF-EAL domain protein. It is demonstrated that AggA, one of TISS components, is indispensable for the secretion of BpfA to cell surface. Thus, DosD regulates the functionality of BpfA, not only by its transcription, but by its secretion through modulating the transcription of TISS.

The TISS in the *bpfA* operon contains three components including AggA encoded by Sputcn32_3594. AggA is a TolC-like protein and has been found to be important for pellicle formation[36]. Pellicles are biofilm-like structures formed at air-liquid interfaces with a high level of oxygen. Transcription of TISS is consistently up-regulated in pellicle cells in *S. oneidensis*. AggA is one of the most up-regulated proteins during biofilm formation[21]. Besides, AggA is also involved in increased biofilm formed by a hyper-aggregating mutant derived from *S. oneidensis*[20]. The roles of AggA in biofilm formation were amply demonstrated in these studies. It is worth noting that AggA is responsible for the secretion of BpfA. Hence, these studies together with us imply the possible role of BpfA in those physiological and morphological observations.

The genetic arrangement of adhesin BpfA and its TISS in *S. putrefaciens* CN32 shares a certain similarity with RTX adhesin and TISS in other bacteria (SI, Figure S7). For some bacteria, the genes encoding for RTX adhesins are also located adjacent to some regulators which are usually able to regulate the functionality of those adhesins. Typically, *lapA* encoding for the largest RTX adhesin in *Pseudomonas fluorescens* is genetically close to the *lapG-lapD* effector system. LapD, a c-di-GMP binding protein, responds to c-di-GMP fluctuation under the control of the PDE RapA. Through the RapA-LapD-LapG-LapA, *P. fluorescens* can quickly respond to a low phosphate stimulus and regulate its biofilm formation. For *S. putrefaciens* CN32, predicted homologues of *lapG-lapD* are located in the *bpfA* operon encoded Sputcn32_3595 and Sputcn32_3597, respectively. Their existence suggests the complex regulation of BpfA localization by c-di-GMP effectors, which warrants further investigations. A homology search revealed that the highly similar genetic arrangements of the *bpfA* operon in S. *putrefaciens* CN32 can be found in many *Shewanella* species (SI, Figure S8). Given the function of the *bpfA* operon revealed here, the *bpfA* operon in other *Shewanella* species might also function in biofilm formation.

In summary, we have elucidated the mechanism of oxygen-regulated biofilm formation by *S. putrefaciens* CN32. However, there is still a missing piece, that is, how the DosD synthesized c-di-GMP alteres the transcription of *bpfA* operon. A transcriptional regulator might be involved, but this needs further validation.

Methods

Strains and growth conditions. *S. putrefaciens* CN32 WT and derivative strains and *Escherichia coli* strains (SI, Table S2) were cultured in Luria-Broth (LB) medium at 28°C and 37°C, respectively. When needed, chemicals were added at the following concentrations: 100 μg/ml diaminopimelic acid, 34 μg/ml chloramphenicol for *E. coli* strains and 10 μg/ml for *Shewanella* strains, 20 μg/ml gentamycin for *E. coli* strains and 10 μg/ml for *Shewanella* strains, kanamycin 50 μg/ml.

Strain and plasmid construction. Genetic manipulations were performed according to the manufacturer's instructions. Kits for DNA and plasmid isolation and purification were purchased from Axygen Co. (Union City, California, USA). Endonucleases, Taq DNA polymerase, reagents for RNA extraction, and kits for reverse transcription and qRT-PCR were purchased from Takara Co. (Dalian, China). Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All primers used for plasmid construction and PCR are listed in Table S3, SI.

Mutants with the in-frame deletion of the desired genes and strains with GFP labeling were constructed essentially as described previously[37] with minor modification (SI, Methods). To create pBGdosD for complementation, the sequence coding for *dosD* and its promoter region was amplified, purified and treated with *Xba* I and *Hind* III. The fragment was cloned into pBBR1MCS5 expression plasmid. The pEGdosD was created to express the His-tagged DosD. The coding sequence of DosD was amplified, restricted with *Nco* I and *Xho* I. The fragment was ligated into pET-

28a(+) and transformed into *E. coli* JM109. After that, the plasmid was re-extracted, sequenced and re-transformed into *E.coli* BL21(DE3) for expression.

Biofilm formation assay and quantification. Overnight cultures were diluted in fresh LB to an OD_{600} of 0.01 and subcultured in glass tubes with shaking at 100 rpm at 28°C. Biofilm that formed on the tube walls was stained using 0.5% crystal violet for 15 min at room temperature, rinsed with 10 mM phosphate buffered saline (PBS pH 7.2) and air-dried. Absorbed crystal violet was dissolved using 33% (v/v) glacial acetic acid and the absorption of the solution at 570 nm was determined for biofilm quantification.

Attachment assay. Cell attachment ability was examined as described earlier[38] with a slight modification. Briefly, coverslips were immersed into the overnight culture of *Shewanella* strains for 15 min with shaking at 150 rpm, and then washed with 10 mM PBS (pH 7.2). The cells adhering to coverslips were viewed under light microscopy Olympus BX51 (Olympus Co., Tokyo, Japan). Average numbers of attached cells from ten randomly chosen fields per coverslip were analyzed using Image-Pro Plus (Media Cybernetics Inc., Bethesda, Maryland, USA).

Expression and purification of DosD. Overnight culture of *E. coli* BL21(DE3) bearing pEGdosD was subcultured in fresh LB medium supplemented with 250 μM 5-aminolevulinic acid hydrochloride and grown at 37°C with shaking at 100 rpm. Induction of expression and purification of DosD was carried out mainly according to manufacturer's instructions with modification (SI, Methods). The expression level of the recombinant DosD was examined by SDS-PAGE and quantitated using the BCA protein assay kit (Beyotime Co., China). The purified recombinant was stored at −70°C until use.

Enzymatic analysis of DosD. The purified His-tagged DosD was diluted in reaction buffer [50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$] to a final concentration of 1–5 μM. To assay the activity of DosD without oxygen binding, deoxy-DosD was prepared by adding 4 mM sodium dithionite to the DosD solutions in an anaerobic chamber (Thermo Inc., Portsmouth, USA) at 30°C. Residual sodium dithionite was removed using a Desalting Gravity Column (Sangon Biotech. Co., Shanghai, China) and reaction buffer. To assay the activity of DosD with oxygen binding, oxy-DosD was prepared by diluting deoxy-DosD in air-saturated reaction buffer.

To initiate the reaction, 50 μM of GTP was added. At given time intervals, aliquots of reaction mixtures were removed, mixed with one-fourth volume of 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) to stop the reaction and then heated at 100°C for 5 min. After centrifugation, 10 μl of the supernatant was analyzed by HPLC system (HPLC1100, Agilent Inc., Santa Clara, USA) equipped with a UV detector set at 254 nm. Separation was achieved on a Zorbax SB C18 column (4.6 mm by 250 mm, Dp, 5 μm; Agilent Techonologies). 9% (v/v) methanol and 91% (v/v) 20 mM triethylamine (pH 7.0) were used as isocratic eluent with a flow rate of 0.8 ml/min. Commercially available c-di-GMP (Biolog Life Science Institute, Bremen, Germany) was used to obtain a calibration curve to access the concentration of c-di-GMP synthesized by the recombinant DosD.

RNA extraction and transcription analysis. Total RNA from cultures was extracted using RNAiso Plus (Takara Co., Dalian, China) according to manufacturer's protocol. The concentration and purity of the final extracted RNA were determined using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The cDNA was synthesized using the PrimeScript RT reagent Kit and qRT-PCR analysis was performed using SYBR Premix Ex Taq (Takara Co., Dalian, China) running on the StepOne real-time PCR system (Applied Biosystems, USA). For each sample, the mean value of triplicate repeats was used to calculate the transcript abundance. For normalization, 16S rRNA in each sample was chosen as the internal standard. The relative cDNA abundance of each gene in the WT was set at 1.0.

Detection of BpfA by Western blot analysis. Subcultures were grown in LB medium shaking at 250 rpm for 8 h. Culture of the WT without GFP labeling was set as the control. Cells in 30 ml subcultures were collected by centrifugation, resuspended in 3 ml of 10 mM PBS and lysed by ultrasonication. Supernatant samples were collected after centrifugation of the lysates and were normalized with protein concentrations determined using a BCA kit before western blot analysis.

Membrane fractions were prepared following a reported protocol[39] with minor modification. Cells in 10 ml of cultures were centrifugated and resuspended in 1 ml SL solution (20% (w/v) sucrose, 1 mg/ml lysozyme, 0.01 M Tris pH8.4) before incubating at 25°C for 1 h. Cells were then collected by centrifugation, resuspended in 200 μl Tris-Mg^{2+} solution (0.01 M $MgCl_2$, 0.01 M Tris pH8.4) and incubated at 25°C for 20 min before centrifugation. The membrane proteins in the supernatant were directly used for western blotting.

Proteins were resolved in SDS-5%PAGE and eletro-transferred onto PVDF membrane in transfer buffer (192 mM Glycine, 24 mM Tris, 20% methanol, 0.05% SDS). GFP-fused BpfA was probed with GFP monoclonal primary antibodies at 1 : 1000 dilution and immuno-coupled with anti-Mouse IgG (H + L)-POD (Roche) according to manufacturer's instructions. BeyoECL Plus (Beyotime Co., China) was used for detection and film images were digitized using Tanon 5500 Gel Imaging System (Tanon Co. Shanghai, China)

c-di-GMP extraction and quantification. Ten ml aerobic cells were harvested by centrifugation after reaching an OD_{600} of 0.8 under aerobic growth. The pellet was washed with 10 mM cold 1× PBS and resuspended in 250 μl of cold (−20°C) extraction solvent (40 : 40 : 20 (v/v/v) methanol-acetonitrile-water). The cell extracts were stored at −20°C for 30 min and then centrifugated. The pellet was re-extracted again using 125 μl of extraction solvent. The extracts were combined and then evaporated at 20°C under a gentle stream of nitrogen gas. The dried extracts were resuspended in 100 μl of ammonium acetate buffer (10 mM ammonium acetate, 0.05% acetate), vortexed and ultrasonicated. After centrifugation, the supernatants were used for quantification of c-di-GMP by LC-MS analysis and the pellets of cell debris were collected for protein quantification.

Aliquots of 15 μl samples were analyzed by LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with Accela 600 pump liquid chromatography (Thermo Fisher Scientific Inc., Waltham, MA, USA). Separation was achieved on a reverse-phase C18 column (Waters Sun Fire; 2.1 by 150 mm; Dp, 5 μm). The column was set at 20°C with a flow rate of 0.2 ml/min. The samples were separated with a 30 min gradient: 0 to 5 min, 0% B; 5 to 10 min 0–20% B; 10 to 15 min, 20–80% B; 15 to 30 min, 80–100% B (A, 20 mM ammonium acetate; B, methanol). The LTQ-Orbitrap mass spectrometer was equipped with an electrospry ion source and performed in positive ion mode. And the others operation condition for LTQ-Orbitrap mass spectrum was performed as follows: ESI source spray voltage 3.5 kv; capillary temperature, 275°C; sheath gas flow rate, 20 arb; auxiliary gas flow rate, 5 arb. Survey MS scans were acquired in the orbitrap analyzer with the resolution set to a value of 60 000 and a scan range from *m/z* 691.00 to 692.00 by selected ion monitoring mode. The data was analyzed using Thermo Xcalibur 2.0.7 (Thermo Fisher Scientific Inc. USA) and the c-di-GMP accuracy quasi-molecular ion$[M + H]^+$ m/z 691.10214 amu ± 5 mmu was selected as the quantitation and confirmation ion mass. The purchased standard c-di-GMP (BIOLOG Life Science Institute, Germany) was used as authentic standard in 10 mM ammonium acetate solution.

1. Heidelberg, J. F. *et al.* Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nat Biotechnol* **20**, 1118–1123 (2002).
2. Hau, H. H. & Gralnick, J. A. Ecology and biotechnology of the genus *Shewanella*. *Annu Rev Microbiol* **61**, 237–258 (2007).
3. Tiedje, J. M. *Shewanella*-the environmentally versatile genome. *Nat Biotechnol* **20**, 1093–1094 (2002).
4. Stoodley, P., Hall-Stoodley, L. & Costerton, J. W. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**, 95–108 (2004).
5. Flemming, H. C. & Wingender, J. The biofilm matrix. *Nat Rev Microbiol* **8**, 623–633 (2010).
6. Lee, Y. K. *et al.* Culture and identification of bacteria from marine biofilms. *J Microbiol* **41**, 183–188 (2003).
7. Barbara, G. M. & Mitchell, J. G. Bacterial tracking of motile algae. *FEMS Microbiol Ecol* **44**, 79–87 (2003).
8. Zhang, M. N., Ginn, B. R., Dichristina, T. J. & Stack, A. G. Adhesion of *Shewanella oneidensis* MR-1 to iron (Oxy)(Hydr)Oxides: microcolony formation and isotherm. *Environ Sci Technol* **44**, 1602–1609 (2010).
9. Nealson, K. H. & Finkel, S. E. Electron flow and biofilms. *MRS Bull* **36**, 380–384 (2011).
10. Logan, B. E. Exoelectrogenic bacteria that power microbial fuel cells. *Nat Rev Microbiol* **7**, 375–381 (2009).
11. Brettar, I., Moore, E. R. B. & Hofle, M. G. Phylogeny and abundance of novel denitrifying bacteria isolated from the water column of the central Baltic Sea. *Microb Ecol* **42**, 295–305 (2001).
12. Liang, Y. L. *et al.* Pellicle formation in *Shewanella oneidensis*. *BMC Microbiol* **10**, 291 (2010).
13. McLean, J. S. *et al.* Oxygen-dependent autoaggregation in *Shewanella oneidensis* MR-1. *Environ Microbiol* **10**, 1861–1876 (2008).
14. Thormann, K. M., Saville, R. M., Shukla, S. & Spormann, A. M. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J Bacteriol* **187**, 1014–1021 (2005).
15. McLean, J. S. *et al.* Investigations of structure and metabolism within *Shewanella oneidensis* MR-1 biofilms. *J Microbiol Methods* **74**, 47–56 (2008).
16. Alam, M., Freitas, T. A. K., Saito, J. A. & Hou, S. B. Globin-coupled sensors, protoglobins, and the last universal common ancestor. *J Inorg Biochem* **99**, 23–33 (2005).
17. Linhartova, I. *et al.* RTX proteins: a highly diverse family secreted by a common mechanism. *FEMS Microbiol Rev* **34**, 1076–1112 (2010).
18. Theunissen, S. *et al.* The 285 kDa Bap/RTX hybrid cell surface protein (SO4317) of *Shewanella oneidensis* MR-1 is a key mediator of biofilm formation. *Res Microbiol* **161**, 144–152 (2010).
19. Satchell, K. J. Structure and function of MARTX toxins and other large repetitive RTX proteins. *Annu Rev Microbiol* **65**, 71–90 (2011).
20. De Windt, W. *et al.* AggA is required for aggregation and increased biofilm formation of a hyper-aggregating mutant of *Shewanella oneidensis* MR-1. *Microbiology* **152**, 721–729 (2006).
21. De Vriendt, K. *et al.* Proteomics of *Shewanella oneidensis* MR-1 biofilm reveals differentially expressed proteins, including AggA and RibB. *Proteomics* **5**, 1308–1316 (2005).
22. Andersen, J. B. *et al.* New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240–2246 (1998).

23. Wan, X. *et al*. Globins synthesize the second messenger bis-(3′–5′)-cyclic diguanosine monophosphate in bacteria. *J Mol Biol* **388**, 262–270 (2009).
24. Vinogradov, S. N. *et al*. Three globin lineages belonging to two structural classes in genomes from the three kingdoms of life. *Proc Natl Acad Sci USA* **102**, 11385–11389 (2005).
25. Tuckerman, J. R. *et al*. An Oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry* **48**, 9764–9774 (2009).
26. Aldridge, P., Paul, R., Goymer, P., Rainey, P. & Jenal, U. Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. *Mol Microbiol* **47**, 1695–1708 (2003).
27. Romling, U. Cyclic di-GMP, an established secondary messenger still speeding up. *Environ Microbiol* **14**, 1817–1829 (2012).
28. Hengge, R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**, 263–273 (2009).
29. Schirmer, T. & Jenal, U. Structural and mechanistic determinants of c-di-GMP signalling. *Nat Rev Microbiol* **7**, 724–735 (2009).
30. Cotter, P. A. & Stibitz, S. c-di-GMP-mediated regulation of virulence and biofilm formation. *Curr Opin Microbiol* **10**, 17–23 (2007).
31. Kader, A., Simm, R., Gerstel, U., Morr, M. & Romling, U. Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica serovar* Typhimurium. *Mol Microbiol* **60**, 602–616 (2006).
32. Rahman, M. *et al*. The role of c-di-GMP signaling in an *Aeromonas veronii* biovar sobria strain. *FEMS Microbiol Lett* **273**, 172–179 (2007).
33. Rakshe, S., Leff, M. & Spormann, A. M. Indirect modulation of the intracellular c-di-GMP level in *Shewanella oneidensis* MR-1 by MxdA. *Appl Environ Microbiol* **77**, 2196–2198 (2011).
34. Plate, L. & Marletta, M. A. Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network. *Mol Cell* **46**, 449–460 (2012).
35. Liu, N. *et al*. Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* **51**, 2087–2099 (2012).
36. Liang, Y. L. *et al*. Transcriptome analysis of pellicle formation of *Shewanella oneidensis*. *Arch Microbiol* **194**, 473–482 (2012).
37. Wang, F., Xiao, X., Ou, H. Y. & Gai, Y. Role and regulation of fatty acid biosynthesis in the response of *Shewanella piezotolerans* WP3 to different temperatures and pressures. *J Bacteriol* **191**, 2574–2584 (2009).
38. Fang, X. & Gomelsky, M. A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. *Mol Microbiol* **76**, 1295–1305 (2010).
39. Martinez-Gil, M., Yousef-Coronado, F. & Espinosa-Urgel, M. LapF, the second largest *Pseudomonas putida* protein, contributes to plant root colonization and determines biofilm architecture. *Mol Microbiol* **77**, 549–561 (2010).

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Author contributions

Y.Y.C., C.W. and H.Q.Y. designed the experiments; C.W., Y.Y.C., L.J.T. and J.C.H. constructed strains and plasmids and conducted other molecular manipulations; L.P.Z. conducted biofilm analysis; X.X.Z. conducted protein expression and activity analysis; H.Y. and X.N.S. conducted the quantification of c-di-GMP by LC-MS; Y.Y.C., C.W. and H.Q.Y. contributed to the planning and coordination of the project; Y.Y.C., W.W.L. and H.Q.Y. wrote and edited the manuscript. All authors contributed to discussion about the results and the manuscript.

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