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 (TSS). Regulation of DosD in aerobic biofilms is heavily dependent on an adhesin BpfA and the TSS. 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. 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. As such, *Shewanella* species have been increasingly recognized as the model system for environmental bacterial metabolism.

Biofilm is a common lifestyle for many bacteria. Microbial cells are embedded in a matrix of self-synthesized extracellular polymeric substances which provides multiple advantages for the survival of the microbial community. *Shewanella* species can form biofilms under diverse environments. For instance, *Shewanella* strains have been isolated from marine biofilms and the surface of green algae. 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. In bioelectrochemical systems, *Shewanella* cells form biofilms on electrode surfaces, which is important for transferring electrons from cells to electrodes.

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. 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. Biofilms formed under aerobic conditions show a hollow and seeding dispersal structure, while a round and densely-packed structure under anaerobic conditions. 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. However, the underlying oxygen sensing and responding mechanisms are mostly unknown.

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, 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 (ΔdosD) was constructed and analyzed. Growth and biofilm formation between wild-type (WT) and ΔdosD 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 ΔdosD 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 Sputcn32_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 ΔdosD 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 ΔdosD. Attachment is important in the initial stage of biofilm formation. Results revealed that the attachment ability of ΔdosD cell on coverslips was significantly reduced compared to WT cells (Figure 1D). Motility assay, however, showed no substantial distinction between the WT and ΔdosD 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 ΔdosD were compared when grown under aerobic and anaerobic conditions, respectively. Under aerobic growth, ΔdosD showed a significant decrease (~40%).
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 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 ΔdosD compared with the WT (Figure 3A).

Differential *bpfA* mRNA levels detected in the WT and ΔdosD 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, 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 ΔdosD *bpfA*::gfp. The bands corresponding to GFP-fused BpfA could be specifically

![Figure 2](https://www.nature.com/scientificreports/)

**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 ΔdosD 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 ns indicates no significant difference (*P* < 0.05).
detected in the samples of GFP-labeled strains but not in the unlabelled-WT. The BpfA level in the ΔdosD 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) apparatus16,17. 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 Sputcn32_3594 is one component of the TISS and has been shown to be involved in the formation of pellicles, biofilms and hyperaggregates2,20,21. Thus, transcription of the aggA gene was also analyzed in this study. Similar to bpfA, the mRNA level of aggA also decreased in ΔdosD 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 ΔaggA were compared with those of the WT. No substantial difference of BpfA in cytoplasm fractions between the ΔaggA bpfA::gfp and the bpfA::gfp was detected. However, BpfA was not detected in the membrane fraction of ΔaggA 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 ΔdosD driven by lac derived P_{A1/04/03} promoter22. ΔdosD 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 ΔdosD expressing AdrA was also significantly increased compared with the ΔdosD 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 ΔdosD, 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 ΔbpfA, ΔdosDΔbpfA, ΔaggA and ΔdosDΔaggA 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 availability19. The rapid detection and response of Shewanella biofilm to oxygen fluctuations has been previously reported19. Cultures of Shewanella cells under aerobic chemostat conditions tend to autoaggregate, which has a similar physiology and morphology to biofilm13. For Shewanella species, oxygen-stimulated biofilm development may help alleviate oxidative stress and/or facilitate the development of hypoxic and even...
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 \(\sim 40\%\) 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 that regulates the c-di-GMP level in the cell.

Through random transposon insertion mutagenesis in S. oneidensis\(^{28}\), 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\(^{29}\). 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\(^{30}\). Besides, AggA is also involved in increased biofilm formation by a hyper-aggregating mutant derived from S. oneidensis\(^{29}\). 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 alters the transcription of bpfA operon. A transcriptional regulator might be involved, but this needs further validation.
Biofilm formation assay and quantification. Overnight cultures were diluted in fresh LB to an OD\textsubscript{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, rinsed with 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 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 μg/mL 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\textsubscript{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 solution 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 × 250 mm, Dp, 5 μm) with a flow rate of 0.8 μM/min. Commerially 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 Reverse Script RT kit (Thermo Fisher Scientific Inc., USA) and the c-di-GMP accuracy quasi-molecular ion[M + H]\textsuperscript{+} m/z 691.1024 µm was selected as the quantitation and 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 691.80 to 693.00 by selected ion monitoring mode. The data was analyzed using the software Xcalibur 2.0.7 (Thermo Fisher Scientific Inc. USA) and the c-di-GMP accuracy quasi-molecular ion[M + H]\textsuperscript{+} m/z 691.1024 µm 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.

Detection of BpfA by Western blot analysis. Subcultures were grown in LB medium shaking at 250 rpm for 8 h. Culture and identification of bacteria from marine biofilms. Nat Biotechnol 8, 623–633 (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.

Additional information

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