Sodium Lactate Negatively Regulates *Shewanella putrefaciens* CN32 Biofilm Formation via a Three-Component Regulatory System (LrbS-LrbA-LrbR)

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ABSTRACT The capability of biofilm formation has a major impact on the industrial and biotechnological applications of *Shewanella putrefaciens* CN32. However, the detailed regulatory mechanisms underlying biofilm formation in this strain remain largely unknown. In the present report, we describe a three-component regulatory system which negatively regulates the biofilm formation of *S. putrefaciens* CN32. This system consists of a histidine kinase LrbS (Sputcn32_0303) and two cognate response regulators, including a transcription factor, LrbA (Sputcn32_0304), and a phosphodiesterase, LrbR (Sputcn32_0305). LrbS responds to the signal of the carbon source sodium lactate and subsequently activates LrbA. The activated LrbA then promotes the expression of *lrbR*, the gene for the other response regulator. The bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) phosphodiesterase LrbR, containing an EAL domain, decreases the concentration of intracellular c-di-GMP, thereby negatively regulating biofilm formation. In summary, the carbon source sodium lactate acts as a signal molecule that regulates biofilm formation via a three-component regulatory system (LrbS-LrbA-LrbR) in *S. putrefaciens* CN32.

IMPORTANCE Biofilm formation is a significant capability used by some bacteria to survive in adverse environments. Numerous environmental factors can affect biofilm formation through different signal transduction pathways. Carbon sources are critical nutrients for bacterial growth, and their concentrations and types significantly influence the biomass and structure of biofilms. However, knowledge about the underlying mechanism of biofilm formation regulation by carbon source is still limited. This work elucidates a modulation pattern of biofilm formation negatively regulated by sodium lactate as a carbon source via a three-component regulatory system in *S. putrefaciens* CN32, which may serve as a good example for studying how the carbon sources impact biofilm development in other bacteria.

KEYWORDS *Shewanella putrefaciens* CN32, biofilm, c-di-GMP, sodium lactate, three-component regulatory system

Biofilms are described as the aggregates of microorganisms in which cells are embedded in a self-produced matrix that are adherent to each other or a surface (1, 2). The ability to form biofilms is a significant factor for the survival of bacteria in stressful environments. Biofilms are produced by bacteria to escape antibiotics, oxidative agents, metals, and nutrient limitation conditions (2–5). Numerous environmental factors can affect biofilm formation through different signal transduction pathways. For example, aminoglycoside antibiotics inhibit the inner membrane phosphodiesterase Arr and increase intracellular bis-(3'-5')-cyclic dimeric GMP (c-di-GMP), which induce biofilm formation in *Pseudomonas aeruginosa* (6). β -Lactam antibiotics promote biofilm

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formation by inducing autolysin-dependent extracellular DNA release in *Staphylococcus aureus* (7, 8). Then two-component regulatory systems (TCSs), the predominant way by which bacteria sense and respond to environment signals, play an important role in the regulation of biofilm development (9). Generally, a TCS contains a sensor histidine kinase and a cytoplasmic cognate response regulator, which transduce extracellular signals into gene expression patterns that facilitate adaptation by bacteria to a specific environment (10). An environmental stimulus activates the kinase or phosphorylase activity of the sensor histidine kinase, which is then transmitted to the cognate response regulator by the transfer of a phosphoryl group (9, 11, 12). Phosphorylation or dephosphorylation modulates the activity of the cognate response regulator by inducing conformational changes (9, 11). Previous studies have demonstrated that the TCS GacS-GacA activates the production of two small noncoding RNAs (RsmZ and RsmY) that can switch *P. aeruginosa* PAO1 between a planktonic lifestyle and a biofilm lifestyle (13–15). Moreover, the TCS EnvZ-OmpR in *Escherichia coli* responds to signals of osmolarity and pH homeostasis and then regulates biofilm formation by controlling flagellum biogenesis and curli production (16). In some cases, one histidine kinase has two cognate response regulators, which constitute a three-component regulatory system. For example, a three-component regulatory system known as RocS1-RocA1-RocR regulates the production of fimbriae during biofilm development in *P. aeruginosa* PAO1 (17–19). RocR has the EAL domain, suggesting that it affects biofilm formation by regulating intracellular c-di-GMP levels (17–19).

Numerous studies have suggested that the second messenger, c-di-GMP, is a core molecule that controls the transition between a planktonic lifestyle and a biofilm state in bacteria (20–22). The current accepted pattern is that high intracellular levels of c-di-GMP induce biofilm formation, whereas low c-di-GMP levels are associated with a motile or planktonic lifestyle (23, 24). The intracellular concentration of c-di-GMP depends on diguanylate cyclase (DGC) and phosphodiesterase (PDE) (24, 25). DGCs containing the GGDEF domain catalyze the synthesis of c-di-GMP from two molecules of GTP, and PDEs containing the EAL domain or HD-GYP domain are involved in the degradation of c-di-GMP to 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) or GMP, respectively (20, 26). Mostly, DGCs and PDEs contain sensory input domains on their N termini such as PAS, GAF, and REC, indicating that several c-di-GMP metabolism enzymes have the capacity to respond to environmental changes (27).

Shewanella putrefaciens, a dissimilatory metal-reducing bacterium, is widely distributed in diverse environments due to its flexible respiration (28). Its respiratory versatility allows it to be applied in some biotechnological and environmental processes (29). *S. putrefaciens* is also an opportunistic pathogen of various aquatic organisms (30–32) and occasionally of humans (33, 34). The biofilm formation of *S. putrefaciens* has a major impact on its biotechnological application. Recent studies have shown that just as for most Gram-negative bacteria, the biofilm formation process of *S. putrefaciens* CN32 is controlled by intracellular c-di-GMP levels (28). DosD with the GGDEF domain has DGC activity *in vivo* and regulates biofilm formation by *S. putrefaciens* CN32 under aerobic conditions (28). BpfA is a key protein to promote biofilm formation in some *Shewanella* species, such as *S. oneidensis* MR-1 and *S. putrefaciens* CN32, and the *bpfA* operon has seven genes that encode a type I secretion system (TISS) for the translocation of BpfA protein (28, 35, 36). c-di-GMP can increase the expression of the *bpfA* operon by weakening the binding between the *bpfA* promoter and its transcriptional repressor, FlrA (37).

In the present report, we describe a three-component regulatory system in *S. putrefaciens* CN32 that consists of a histidine kinase (Sputcn32_0303) and two cognate response regulators, including a transcription factor (Sputcn32_0304) and a PDE with an EAL domain (Sputcn32_0305). Sputcn32_0303 stimulates one of its cognate response regulators, Sputcn32_0304, which subsequently promotes the transcription of *Sputcn32_0305*, whereas Sputcn32_0305 degrades c-di-GMP in cells, which, in turn, regulates biofilm formation by *S. putrefaciens* CN32. Finally, we found that sodium

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5α	<i>endA1 hsdR17(r⁻ m⁺) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)U169 deoR(Φ80dlacZ ΔM15)</i>	Lab stock
BL21/DE3	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS(r_B⁻ m_B⁻) gal λ(DE3)</i>	Lab stock
S17-1	<i>thi endA recA hsdR</i> with RP4-2-Tc::Mu-Km::Tn7 integrated in the chromosome; Sm ^r	60
UQ3021	DH5α/λpir	55
UQ3022	UQ3021/pRL27; Km ^r	55
XL1-Blue	<i>endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[Tn10 proAB⁺ lac^qΔ(lacZ)M15] hsdR17(r_K⁻ m_K⁺) Km^r Tc^r Cm^r</i>	Lab stock
BTH101	F ⁻ <i>cya-99 araD139 galE15 galk16 rpsL1 (Str^r) hsdR2 mcrA1 mcrB1</i>	61
<i>S. putrefaciens</i>		
CN32	Wild type	28
Δ0303	CN32 Δ <i>Sputcn32_0303</i> :: <i>aacC1</i> Gm ^r	This study
Δ0304	CN32 Δ <i>Sputcn32_0304</i> reserving 144 bp after start codon	This study
Δ0305	CN32 Δ <i>Sputcn32_0305</i>	This study
Δ0303Δ0304	CN32 (Δ <i>Sputcn32_0303</i> Δ <i>Sputcn32_0304</i>):: <i>aacC1</i> Gm ^r	This study
Δ0303Δ0305	CN32 (Δ <i>Sputcn32_0303</i> :: <i>aacC1</i>)Δ <i>Sputcn32_0305</i> Gm ^r	This study
Δ0304Δ0305	CN32 (Δ <i>Sputcn32_0304</i> reserving 144 bp after start codon)Δ <i>Sputcn32_0305</i>	This study
Δ0303Δ0304Δ0305	CN32 Δ <i>Sputcn32_0303</i> Δ <i>Sputcn32_0304</i> Δ <i>Sputcn32_0305</i>	This study
C-0303	Δ0303 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0303</i> ; Km ^r	This study
C-0304	Δ0304 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0304</i> ; Km ^r	This study
C-0305	Δ0305 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0305</i> ; Km ^r	This study
C-0304-D54A	C-0304 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0304 D54A</i> ; Km ^r	This study
C-0304-D54E	C-0304 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0304 D54E</i> ; Km ^r	This study
C-0305-D56A	C-0305 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0305 D56A</i> ; Km ^r	This study
C-0305-D56E	C-0305 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0305 D56E</i> ; Km ^r	This study
C-0305-E179A	C-0305 carrying complement pBBR1MCS-2-P _{<i>aacC1</i>} - <i>Sputcn32_0305 E179A</i> ; Km ^r	This study
Plasmids		
pUX19	Suicide plasmid for CN32; Km ^r	62
pUCGm	pUC19 derivative containing <i>aacC1</i> (encoding Gm ^r); Ap ^r Gm ^r	63
pK19mobsacB	Suicide plasmid for CN32; <i>sacB</i> (modified from <i>B. subtilis</i>) <i>lacZ</i> Km ^r	64
pBBR1MCS-2	Broad-host-range plasmid; Km ^r <i>lacZ</i>	65
pRK2013	Helper plasmid in matings; Km ^r	66
pET-28a(+)	Vector for protein overexpression in <i>E. coli</i> ; Km ^r	Novagen
pUT18c	Cloning and expression vector; encodes the T18 fragment (amino acids 225–399 of CyaA); Ap ^r	61
pKT25	Cloning and expression vector, encodes the T25 fragment (amino acids 1–224 of CyaA); Km ^r	61
pRL27	Contains mini-Tn5 transposon (oriR6K) delivery vector; Km ^r	55
pBKmini-Tn7-Gm-Gfp	Delivery vector for mini-Tn7-Gm- <i>gfp</i> ; Gm ^r	67
pBKmini-Tn7-Strep-Gfp	Delivery vector for mini-Tn7-Strep- <i>gfp</i> ; Sm ^r	67

^a*aacC1*: the arrow above *aacC1* indicates the transcription direction of *aacC1* gene. Δ*Sputcn32_0303*::*aacC1*, the transcription direction of *aacC1* was consistent with that of original *Sputcn32_0303*.

lactate negatively regulated biofilm formation via the three-component regulatory system.

RESULTS

Histidine kinase *Sputcn32_0303* negatively regulates biofilm formation by *S. putrefaciens* CN32. Approximately 10,000 kanamycin-resistant *S. putrefaciens* CN32 mini-Tn5 transposon insertion mutants were screened for altered biofilm formation ability using the plate assay. These screening experiments and subsequent identification led to the selection of 56 insertion mutations, 2 of which were inserted into a different locus in *Sputcn32_0303*; and both insertion mutants expressed a highly robust biofilm phenotype. Sequence analyses indicated that *Sputcn32_0303* is a histidine kinase that had one transmembrane region (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). To elucidate the function of the *Sputcn32_0303*, a gene replacement mutant, Δ0303 (Table 1), was constructed. The biofilm biomass of the Δ0303 mutant increased significantly compared to that of the wild-type (WT) strain, and the biofilm of the complementation strain C-0303 was restored to the WT level (Fig. 1A), indicating that

FIG 1 (A and B) Biofilm formation (A) and cell growth (B) of the WT and $\Delta 0303$ and C-0303 strains. (C and D) Biofilm formation (C) and cell growth (D) of the WT and $\Delta 0304$ and C-0304 strains. (E) Biofilm formation of the WT, $\Delta 0305$, C-0305, and C-0305-E179A strains. (F) Cell growth of the WT, $\Delta 0305$, and C-0305 strains. All the strains were cultured in MM1 medium. Values are means $\pm$ standard deviations ($n = 16$ for biofilm formation and $n = 3$ for cell growth).

the increase of biofilm in the $\Delta 0303$ mutant was due solely to the *Sputcn32_0303* deletion. The growth rates of the three strains showed no significant differences (Fig. 1B), which suggested that the altered biofilm was not caused by the change of cell growth. Taken together, our results show that the presence of *Sputcn32_0303* negatively regulated biofilm formation by *S. putrefaciens* CN32.

***Sputcn32_0303*, *Sputcn32_0304*, and *Sputcn32_0305* encode members of a three-component regulatory system.** We next analyzed the genes adjacent to *Sputcn32_0303*. The genomic arrangement of these three genes is shown in Fig. 2A. The upstream gene *Sputcn32_0304*, which encodes a protein that similarly acts as a transcription factor of TCSs, is cotranscribed with *Sputcn32_0303* (two open reading frames [ORFs] have a 2-bp overlap), and the gene *Sputcn32_0305*, which encodes a protein containing an EAL domain, is transcribed in the opposite direction to the *Sputcn32_0304*-*Sputcn32_0303* operon. Domains of these proteins are predicted according to <http://smart.embl-heidelberg.de> and are shown in Fig. 2B. The genomic arrangement and

FIG 2 Sputcn32_0303, Sputcn32_0304, and Sputcn32_0305 constitute a three-component regulatory system. (A) Genomic arrangement of *Sputcn32_0303*, *Sputcn32_0304*, and *Sputcn32_0305*. The numbers in arrows are the gene sizes, and the numbers below the arrows are the lengths of intergenic sequences (a minus indicates that two ORFs overlap). The small bent arrows are the promoters of the *Sputcn32_0304-0303* operon and *Sputcn32_0305*. (B) The protein domains of *Sputcn32_0303*, *Sputcn32_0304*, and *Sputcn32_0305* are predicted according to <http://smart.embl-heidelberg.de>. The H1 is the autophosphorylation domain in histidine kinase, and D1, H2, and D2 are the transfer domains of a phosphoryl group. (C) Bacterial two-hybrid experiments. N, negative control with the empty vector pUT18C and pKT25 in BTH101; SxA, positive control with pKT25-RocS2HPT and pUT18C-RocA2D2 in BTH101; 3x4, pKT25-0303HPT and pUT18C-0304D2 in BTH101; 3x5, pKT25-0303HPT and pUT18C-0305D2 in BTH101; 3xA, negative control with pKT25-0303HPT and pUT18C-RocA2D2 in BTH101. The interactions were identified after 24 h of incubation at 30°C.

protein domains of the three genes are similar to the three-component regulatory system RocS1-RocA1-RocR in *Pseudomonas aeruginosa* PAO1 (17). To confirm that the products of the three genes *Sputcn32_0303*, *Sputcn32_0304*, and *Sputcn32_0305* constitute a three-component regulatory system, we performed bacterial two-hybrid experiments. In previous studies, a two-component regulatory system was verified by analyzing the interaction between the phosphoryl group transfer domains HPT and D2 (17, 38). In this study, the results of bacterial two-hybrid experiments showed that *Sputcn32_0303*-HPT directly interacted with *Sputcn32_0304*-D2 and *Sputcn32_0305*-D2 (Fig. 2C), indicating that *Sputcn32_0303*, *Sputcn32_0304*, and *Sputcn32_0305* act as a three-component regulatory system.

The three-component regulatory system is associated with biofilm formation.

In *Pseudomonas aeruginosa* PAO1, the two response regulators RocA1 and RocR of the RocS1-RocA1-RocR three-component regulatory system have opposite effects on the expression of the fimbrial gene (17). In this study, we found that *Sputcn32_0303* could negatively regulate biofilm formation, so we next investigated the effects of the two cognate response regulators, *Sputcn32_0304* and *Sputcn32_0305*, on biofilm formation by *S. putrefaciens* CN32. Two single mutants of *Sputcn32_0304* and *Sputcn32_0305*, $\Delta 0304$ and $\Delta 0305$, were constructed. The deletion mutant of *Sputcn32_0304* was constructed according to previous studies (39–41), which can avoid the polar effect on expression of the downstream gene *Sputcn32_0303* and facilitate the investigation on its own expression changes. The biofilm biomasses of both single mutants were significantly increased compared to that of the WT strain, and biofilms of both complementation strains C-0304 and C-0305 were restored to the WT level (Fig. 1C and E), whereas their growth rates did not substantially differ (Fig. 1D and F). These results indicated that the three-component regulatory system negatively regulated biofilm formation under static conditions.

We next used confocal laser scanning microscopy (CLSM) to monitor the structures of live biofilms that were generated by the WT strain and the three single-mutants $\Delta 0303$, $\Delta 0304$, and $\Delta 0305$ in the flow cell chambers after 2 days of culture in MM1 medium. The WT biofilm stayed at the initial attachment stage and did not form microcolonies (Fig. 3A). However, all the three mutants progressed to maturity and developed into towering and mushroom-like protrusions (Fig. 3B to D). All these results

FIG 3 Biofilm formation by the WT strain (A), the $\Delta 0303$ mutant (B), the $\Delta 0304$ mutant (C), and the $\Delta 0305$ mutant (D) under the hydrodynamic condition monitored by CLSM at 48 h. (E) Biofilms formed by the WT and the seven mutants under the static condition; values for biofilm formation are means $\pm$ standard deviations ($n = 8$). All the strains were cultured in MM1 medium.

indicated that the three-component regulatory system negatively regulates biofilm development by *S. putrefaciens* CN32 under both static and hydrodynamic conditions.

Moreover, we constructed double mutants and a triple mutant with mutations of these three genes. The biofilm biomasses formed by all seven mutants were essentially similar and significantly higher than that of the WT strain (Fig. 3E), indicating that the three-component regulatory system was involved in the same signal transduction pathway.

Sputcn32_0305 has PDE activity *in vivo*. We next separately investigated the functions of these three proteins in the three-component regulatory system. Sputcn32_0305, a response regulator of the three-component regulatory system, contains an EAL domain that may possess PDE activity to degrade c-di-GMP in cells. To determine whether the Sputcn32_0305 impacts the intracellular c-di-GMP level, the intracellular c-di-GMP concentrations in the WT and $\Delta 0305$ mutant were examined by liquid chromatography-mass spectrometry (LC-MS). The intracellular c-di-GMP concentration of $\Delta 0305$ increased about 2-fold compared to that of the WT strain, and the c-di-GMP concentra-

tion of the complementation strain C-0305 was restored to the WT level (Fig. 4). Moreover, to genetically test the role of *Sputcn32_0305* as a PDE, a site-directed mutant, C-0305-E179A, was constructed to change the EVL catalytic motif to AVL. The results showed that both biofilm biomass and intracellular c-di-GMP level of C-0305E179A were not restored to the WT levels (Fig. 1E and 4), indicating that *Sputcn32_0305* did have PDE activity *in vivo*. A previous study suggested that a 1-fold increase of the intracellular c-di-GMP level could significantly enhance biofilm formation by *S. putrefaciens* CN32 (28). Thus, the more robust biofilms of the $\Delta 0305$ mutant than of the WT strain (Fig. 1E and 3D) were due to its high intracellular c-di-GMP levels.

Sputcn32_0304 directly promotes the expression of *Sputcn32_0305*. *Sputcn32_0304*, the second response regulator in the three-component regulatory system, has a predicted effector domain with a helix-turn-helix (HTH) motif that is essentially similar to the HTH domains in the LuxR/GerE family transcriptional regulators. In previous studies, a transcription factor was shown to regulate the expression of its own gene (39, 42). To determine whether *Sputcn32_0304* directly regulates the expression of its own gene and *Sputcn32_0305*, an electrophoretic mobility shift assay (EMSA) was performed. The probe *0304_0305_int* was designed to cover the entire *Sputcn32_0304* and *Sputcn32_0305* intergenic region (367 bp [Fig. 2A]), which contained the bidirectional promoter region of *Sputcn32_0304* and *Sputcn32_0305*. The EMSA result showed that His₆-0304 could bind to probe *0304_0305_int* (Fig. 5A). Moreover, this binding was specific, as it could be outcompeted by adding excess (300-fold) unlabeled specific probe *0304_0305_int* (Fig. 5A, lane S), and a 300-fold excess of unlabeled nonspecific competitor DNA did not abolish the delayed band (Fig. 5A, lane N).

To investigate the effect of *Sputcn32_0304* deletion on the expression of its own operon and *Sputcn32_0305*, the transcriptional profiles of the *Sputcn32_0303*, *Sputcn32_0304*, and *Sputcn32_0305* genes in the WT and $\Delta 0304$ mutant were analyzed by real-time reverse transcription-PCR (RT-PCR). Levels of expression of both *Sputcn32_0304* and *Sputcn32_0303* in the $\Delta 0304$ mutant increased to 1.5-fold that in the WT strain (Fig. 5B), indicating that *Sputcn32_0304* negatively regulated the transcription of the *Sputcn32_0304-0303* operon. On the other hand, the expression of *Sputcn32_0305* in the $\Delta 0304$ mutant was decreased significantly compared to that in the WT strain (Fig. 5C), which suggested that the presence of *Sputcn32_0304* promoted *Sputcn32_0305* transcription in *S. putrefaciens* CN32.

To analyze the putative mechanism of the opposite effect of *Sputcn32_0304* on the expression of its own operon and *Sputcn32_0305*, the transcriptional start sites (TSSs) of *Sputcn32_0304* and *Sputcn32_0305* were determined by 5' rapid amplification of cDNA end (RACE) PCR analysis. The TSS of *Sputcn32_0304* was 16 nucleotides (nt) away from that of *Sputcn32_0305*. The two TSSs were located on the opposite strand and between the putative -10 and -35 regions of the other gene (Fig. 6A). Moreover, as we failed to find the *Sputcn32_0304* binding site by footprinting, EMSAs were performed using *Sputcn32_0304* and different lengths of DNA probes (P1 to P6) (Fig. 6B). *Sputcn32_0304* could bind to probes P1, P3, and P5, but no shifted bands were observed with probes P2, P4, and P6 (Fig. 6C). These results suggested that the putative *Sputcn32_0304* binding site was located downstream of the *Sputcn32_0304* TSS. Thus, we speculated that the negative autoregulation of *Sputcn32_0304* may be due to slowing down its own transcription elongation, but the promotion of *Sputcn32_0304* to *Sputcn32_0305* transcription may be due to altering DNA conformation or recruiting RNA polymerase.

The negative effects of *Sputcn32_0304* and *Sputcn32_0305* on biofilm depend on their phosphorylation state *in vivo*. Mostly, the activity of the response regulator in two-component regulatory system depends on their phosphorylation state *in vivo* (13). On the other hand, although some response regulators without phosphorylation could bind to their cognate recognition sequences *in vitro*, the phosphorylation state may enhance this binding (43, 44). To determine whether the phosphorylation state enhances the binding between *Sputcn32_0304* and probe *0304_0305_int* *in vitro*,

FIG 4 Intracellular c-di-GMP concentrations of the WT, Δ0305, C-0305, and C-0305-E179A strains at 6 h. All the strains were cultured in MM1 medium. Error bars indicate standard deviations for 3 samples.

phosphorylated His₆-0304 was used for EMSAs. The phosphorylation state of His₆-0304 was changed using the following two methods. In the first method, to mimic and prevent phosphorylation, two proteins with site-directed mutagenesis of the phosphorylation residues, His₆-0304D54E and His₆-0304D54A, were purified. In the second method, His₆-0304 was phosphorylated by incubation with acetyl phosphate (AcP). The EMSAs showed that the binding between the phosphorylated protein and probe DNA was not enhanced by either method (Fig. 7), indicating that phosphorylated Sputcn32_0304 did not exhibit higher affinity for the promoter than dephosphorylated protein *in vitro*.

To determine whether the phosphorylation state impacts the activities of Sputcn32_0304 *in vivo*, we construct site-directed mutants of the phosphorylation

FIG 5 Sputcn32_0304 directly regulates the expression of *Sputcn32_0304-0303* operon and *Sputcn32_0305*. (A) EMSA of His₆-0304 binding to the probe 0304_0305_int (*Sputcn32_0304-Sputcn32_0305* intergenic region, 367 bp). Lane –, labeled probe was incubated in the absence of His₆-0304. The triangle above the lanes indicates that labeled probe 0304_0305_int was incubated with 60 nM, 120 nM, 240 nM, and 360 nM His₆-0304. The binding specificity was confirmed by competitive assays with a 300-fold excess of unlabeled specific probe 0304_0305_int (lane S) and a 300-fold excess of unlabeled nonspecific competitor DNA (probe *recA*) (lane N). (B) Relative levels of expression of *Sputcn32_0304* and *Sputcn32_0303* in the WT strain and the Δ0304 mutant after culturing in MM1 medium for 6 h. (C) Relative levels of expression of *Sputcn32_0305* in the WT, Δ0304 mutant, and Δ0303 mutant after culturing in MM1 medium for 6 h. Error bars indicate standard deviations for 3 samples.

FIG 6 (A) Nucleotide sequences of the *Sputcn32_0304*-*Sputcn32_0305* intergenic region. Numbers on the left of the sequences are distances (in nucleotides) from the TSS of *Sputcn32_0304*. Numbers on the right of the sequences are distances (in nucleotides) from the TSS of *Sputcn32_0305*. Red bent arrows, TSS of *Sputcn32_0304*; red areas, putative -10 and -35 regions of *Sputcn32_0304*; green bent arrows, TSS of *Sputcn32_0305*; green areas, putative -10 and -35 regions of *Sputcn32_0305*; shaded areas, translational start codons. (B) Locations of probes P1 to P6. (C) EMSAs of His₆-0304 (240 nM) with probes P1 to P6.

residues, C-0304-D54A and C-0304-D54E. Then the transcriptional profiles of the *Sputcn32_0305* gene in the site-directed mutants were analyzed by real-time RT-PCR. The results showed that the expression of *Sputcn32_0305* was decreased significantly in the $\Delta 0304$ mutant and was restored to the WT level in C-0304 and C-0304-D54E. However, the expression of *Sputcn32_0305* in C-0304-D54A could not be restored to the WT level and showed a low level similar to that in the $\Delta 0304$ mutant (Fig. 8A), indicating

FIG 7 (A) EMSAs of His₆-0304D54A binding to the probe 0304_0305_int. (B) EMSAs of His₆-0304D54E binding to the probe 0304_0305_int. The triangle above the lanes indicates that labeled probe 0304_0305_int was incubated with 60 nM, 120 nM, 240 nM, and 360 nM protein. (C) Binding of probe 0304_0305_int and His₆-0304 (240 nM) incubated with AcP. Labeled probe was incubated in the absence of His₆-0304 (lane 1). His₆-0304 was incubated with phosphorylation buffer in the absence of AcP at 25°C for 60 min (lane 2), in the presence of 12.5 mM AcP at 25°C for 60 min (lane 3), in the absence of AcP at 25°C for 120 min (lane 4), and in the presence of 12.5 mM AcP at 25°C for 120 min (lane 5).

that the transcriptional regulatory activity of Sputcn32_0304 depended on its phosphorylation state *in vivo*. To further confirm that the activities of the two response regulators depend on their phosphorylation state *in vivo*, we construct site-directed mutants of Sputcn32_0305, including dephosphorylation mutant C-0305-D56A and phosphorylation mutant C-0305-D56E. Both phosphorylation mutants, C-0304-D54E and C-0305-D56E, produced biofilms similar to that of the WT strain. In contrast, neither of the dephosphorylation mutants, C-0304-D54A and C-0305-D56A, generated biofilms similar to that of the Δ 0304 mutant (Fig. 8B and C). These results indicated that although the phosphorylation state could not enhance the binding between Sputcn32_0304 and its cognate recognition sequence *in vitro*, the negative effects of Sputcn32_0304 and Sputcn32_0305 on biofilm formation depend on their phosphorylation state *in vivo*. In addition, in the three-component regulatory system, Sputcn32_0303 directly interacted with its two cognate response regulators, Sputcn32_0304 and Sputcn32_0305 (Fig. 2C), suggesting that the phosphorylation states of Sputcn32_0304 and Sputcn32_0305 depend on their cognate histidine kinase, Sputcn32_0303.

Sodium lactate negatively regulates biofilm formation via Sputcn32_0303.

During our experiments, we found that the biofilms of the WT strain and the Δ 0303 mutant were similar when the two strains were cultured in LB medium (data not shown), whereas distinct biofilm differences were observed between the WT strain and the Δ 0303 mutant after culturing in MM1 medium supplemented with 20 mM sodium lactate as the sole carbon source. These results suggested that MM1 medium contains the component which negatively regulates biofilm formation via Sputcn32_0303. A previous study showed that when *E. coli* is cultured under anaerobic conditions, the PAS domain of the histidine kinase CitA senses the carbon source sodium citrate (45). In the present study, to validate whether Sputcn32_0303 responds to sodium lactate,

FIG 8 (A) Relative levels of expression of *Sputn32_0305* by the WT, $\Delta 0304$, C-0304, C-0304-D54A, and C-0304-D54E strains lactate. Error bars indicate standard deviations for 3 samples. (B and C) Biofilms formed by the WT, $\Delta 0304$, C-0304, C-0304-D54A, and C-0304-D54E strains (B) and the WT and $\Delta 0305$, C-0305, C-0305-D56A, and C-0305-D56E strains (C). All strains were cultured in MM1 medium supplemented with 20 mM sodium lactate. Error bars indicate standard deviations for 16 samples.

a concentration gradient assay of sodium lactate was performed. The WT strain and the $\Delta 0303$ mutant were cultured in MM1 medium supplemented with 5 mM, 10 mM, 20 mM, and 30 mM sodium lactate, while both strains cultured in MM1 medium with 1.7 g/liter of yeast extract but without sodium lactate were used as controls. As shown in Fig. 9A, there was a positive correlation between the concentration of sodium lactate and the inhibition of biofilm formation in the WT, while the biofilms formed by the $\Delta 0303$ mutant that were cultured with different sodium lactate concentrations showed

FIG 9 Sodium lactate negatively regulates biofilm formation. (A) Biofilms formed by the WT strain and Δ0303 mutant in MM1 medium with different concentrations sodium lactate (5 mM, 10 mM, 20 mM, and 30 mM) and in the control medium (MM1 medium with 1.7 g/liter of yeast extract without sodium lactate) at 6 h. Error bars indicate standard deviations for 8 samples. (B) Growth profiles of the WT strain and Δ0303 mutant in MM1 medium with 5 mM and 30 mM sodium lactate. SL, sodium lactate; YE, yeast extract. Error bars indicate standard deviations for 3 samples. (C and D) Biofilms formed by the WT strain and Δ0303 mutant in MM1 medium with different concentrations (10 mM, 20 mM, and 30 mM) of sodium propionate (C) and sodium acetate (D) as the sole carbon source.

similar features. However, in the control medium (MM1 medium with 1.7 g/liter of yeast extract but without sodium lactate), the WT strain and the Δ0303 mutant generated biofilms with similar features. These results indicated that sodium lactate negatively regulated biofilm formation by the WT strain via *Sputcn32_0303*. Moreover, the levels of cell growth of the WT strain and the Δ0303 mutant in the same concentration of sodium lactate were similar, and a high concentration of sodium lactate promoted the growth of planktonic cells of both strains (Fig. 9B), suggesting that the negative effects of sodium lactate on the WT biofilm were not caused by the changes in cell growth.

To evaluate whether other common carbon sources that were similar to sodium lactate negatively regulate biofilm via *Sputcn32_0303*, we performed biofilm assays using MM1 medium supplemented with different concentrations of sodium citrate, sodium propionate, and sodium acetate. We found that although sodium citrate is a main carbon source for the biofilm formation of *P. aeruginosa*, *S. putrefaciens* CN32 cannot utilize sodium citrate as a carbon source. As shown in Fig. 9C and D, no significant differences in biofilm formation were apparent between the WT strain and the Δ0303 mutant when both strains were cultured in the presence of sodium propionate or sodium acetate as the carbon source, indicating that *Sputcn32_0303* may only respond to sodium lactate.

Proposed model for sodium lactate negatively regulating biofilm formation.

Based on the above-described results, we propose a regulation model in which sodium lactate negatively regulates biofilm formation of *S. putrefaciens* CN32 via the three-component regulatory system (Fig. 10A). The histidine kinase, *Sputcn32_0303*, responds to sodium lactate signal and activates one of the cognate response regulators, *Sputcn32_0304*, which subsequently promotes the expression of *Sputcn32_0305*. *Sputcn32_0305* has PDE activity *in vivo* that can degrade intracellular c-di-GMP, thereby leading to the negative regulation of biofilm formation by *S. putrefaciens* CN32.

FIG 10 (A) Proposed model for sodium lactate negatively regulating biofilm formation. The histidine kinase, *Sputcn32_0303*, responded to the signal of sodium lactate and subsequently activated its cognate response regulators, *Sputcn32_0304* and *Sputcn32_0305*, thereby negatively regulating biofilm formation via decreasing the intracellular c-di-GMP levels. Straight arrows indicate activation, and T arrows indicate inhibition. Solid arrows indicate direct regulation, and dashed arrows indicate indirect regulation. (B) Intracellular c-di-GMP concentrations of the WT strain, the Δ0304 mutant, and the Δ0303 mutant at 6 h. All the strains were cultured in MM1 medium supplemented with 20 mM sodium lactate. (C) Relative expression of *Sputcn32_0305* in the WT and Δ0303 strains after 6 h of culture in MM1 medium with different concentrations of sodium lactate (5 mM, 20 mM, and 30 mM) or with 1.7 g/liter of yeast extract without sodium lactate (the control medium). Error bars indicate standard deviations for 3 samples.

To further confirm this model, we analyzed the intracellular c-di-GMP level in the WT, Δ0303, and Δ0304 (Fig. 10B). The result showed that the intracellular c-di-GMP levels in the two mutants were similar and significantly higher than that of the WT strain. Moreover, the levels of expression of *Sputcn32_0305* in Δ0303 and Δ0304 were similar and were decreased significantly compared to that of the WT strain (Fig. 5C), indicating the *Sputcn32_0303* is essential for *Sputcn32_0304* to promote the expression of *Sputcn32_0305*. These results suggested that the transcriptional regulatory activity of *Sputcn32_0304* was triggered by its cognate histidine kinase, *Sputcn32_0303*.

The transcription of *Sputcn32_0305* in the WT strain was significantly upregulated with higher concentrations of sodium lactate (Fig. 10C), while the transcription of *Sputcn32_0305* in the Δ0303 mutant, which was cultured with different sodium lactate concentrations, was similar (Fig. 10C). However, the levels of expression of *Sputcn32_0305* in the WT strain and the Δ0303 mutant were similar in the control medium (MM1 medium with 1.7 g/liter of yeast extract but without sodium lactate) (Fig. 10C). These results suggested that sodium lactate regulated the transcription of *Sputcn32_0305* via *Sputcn32_0303*. We hence designate *Sputcn32_0303* (sensor),

Sputcn32_0304 (activator), and Sputcn32_0305 (regulator) LrbS, LrbA, and LrbR, respectively, where “Lrb” stands for sodium lactate-dependent repressor for biofilm formation.

DISCUSSION

The present work revealed a three-component regulatory system, Sputcn32_0303-Sputcn32_0304-Sputcn32_0305 (LrbS-LrbA-LrbR), that responds to the presence of a carbon source, sodium lactate, and regulates biofilm formation by *S. putrefaciens* CN32. In *Pseudomonas aeruginosa* PAO1, the three-component regulatory system RocS1-RocA1-RocR has been found to be able to regulate biofilm formation via regulating fimbrial gene expression (17). Its two response regulators, RocA1 and RocR, exhibit opposite effects on the expression of the fimbrial gene. However, the signals sensed by the histidine kinase, RocS1, are unknown. In *S. putrefaciens* CN32, in contrast to RocA1 and RocR, response regulators Sputcn32_0304 and Sputcn32_0305 showed similar negative effects on biofilm formation as Sputcn32_0304 promoted the expression of *Sputcn32_0305*. Moreover, we found that the histidine kinase, Sputcn32_0303, responded to the signal of sodium lactate.

Sodium lactate can be used as a carbon source by *Shewanella* species during their planktonic growth and biofilm formation (46, 47). Carbon sources are critical nutrients for bacterial growth, and their concentrations and types significantly influence the biomass and structure of biofilms (47–50). For example, the deprivation of the carbon source can trigger the production of extracellular polymeric substances (EPS) in the biofilm of *Bacillus subtilis* (48). In addition, the three-dimensional (3D) structure of biofilms formed by *P. aeruginosa* under hydrodynamic conditions is totally dependent on the type of carbon source (49, 51). In present study, we found that the carbon source sodium lactate negatively regulated biofilm formation of *S. putrefaciens* CN32. Similar negative regulation of biofilm formation by sodium lactate has been observed in *S. oneidensis* MR-1 (47), but the underlying regulation pathway has not been revealed. We found that Sputcn32_0303 responded to sodium lactate, which subsequently activated its cognate response regulators (Fig. 10A), thereby negatively regulating biofilm formation via decreasing the intracellular c-di-GMP levels in *S. putrefaciens* CN32.

The second messenger molecule, c-di-GMP, promotes biofilm formation and inhibits motility (23, 52). As a core regulatory molecule, intracellular c-di-GMP plays an important role throughout all stages of biofilm development in many bacteria, such as *Shewanella* and *Pseudomonas* (24, 53). BpfA is an adhesion protein and contributes greatly to biofilm formation by *Shewanella* species (28, 35, 36). Recently, Cheng et al. found that c-di-GMP can weaken the binding between the promoter of the *bpfA* operon and its transcriptional repressor FlrA, thereby increasing the expression of the *bpfA* operon (37). The present study showed that the PDE Sputcn32_0305 in the three-component regulatory system decreased the intracellular c-di-GMP concentration (Fig. 4). And we found that the expression of *bpfA* was increased 2- to 6-fold in the Δ 0305 mutant compared to the WT strain (data not shown), indicating that the three-component regulatory system responds to the carbon source sodium lactate, degrades intracellular c-di-GMP, and negatively regulates biofilm formation by decreasing the expression of the *bpfA* operon. In addition, we found that the presence of Sputcn32_0305 inhibited microcolony formation in *S. putrefaciens* CN32 (Fig. 3A to D). Previous studies have shown that the genes involved in polysaccharide synthesis (54) and twitching motility controlled by type IV pili (49) are essential to microcolony formation by *P. aeruginosa*. However, studies that aim to identify genes that are related to microcolony formation by *S. putrefaciens* CN32 are limited. Therefore, to identify the target genes modulated by c-di-GMP levels that are regulated by the three-component regulatory system, we will perform transcriptome sequencing (RNA-seq) assays to establish the differential expression profiles between the WT strain and the Δ 0305 mutant in future work.

MATERIALS AND METHODS

Bacterial strains and growth conditions. WT *Shewanella putrefaciens* CN32 and its derivative strains were cultured at 28°C in lysogeny broth (LB) or modified M1 medium (MM1) (pH 7) which consisted of 30 mM HEPES, 7.5 mM NaOH, 28.04 mM NH₄Cl, 1.34 mM KCl, 4.35 mM NaH₂PO₄, 0.68 mM CaCl₂ supplemented with trace amounts of minerals, vitamins, and amino acids, and 20 mM sodium lactate added as the sole carbon source (46). Cell growth was determined in MM1 medium without CaCl₂. *E. coli* cells were cultured in LB medium at 37°C. When necessary, the medium was supplemented with 7.5 µg/ml of gentamicin or 50 µg/ml of kanamycin for *S. putrefaciens* CN32 and 15 µg/ml of gentamicin or 50 µg/ml of kanamycin for *E. coli*. The strains and plasmids constructed or used in this study are listed in Table 1, and the primers used are listed in Table 2. Kits used for isolation and purification of DNA were purchased from Tiangen and Genstar, respectively. Enzymes were purchased from New England Biolabs and Thermo Scientific.

Transposon mutagenesis. Transposon mutagenesis was performed by means of biparental mating between an *E. coli* UQ3022 donor (harboring pRL27, the suicide vector, and carrying mini-Tn5-Km) (55) and the receptor bacterium, *S. putrefaciens* CN32. *E. coli* UQ3022 and *S. putrefaciens* CN32 were cultured in LB medium overnight. The cells were harvested and washed twice using LB medium. Approximately 50-µl mixtures of each strain were spot inoculated on LB agar plates. After 5 h of incubation at 28°C, the cells were scraped off and resuspended using 1 ml of LB medium. A transposon insertion library was generated by plating 0.1 ml of the cell suspension per selective LB agar plate supplemented with 20 µg/ml of tellurite and 50 µg/ml of kanamycin. Then the plates were incubated at 28°C for 36 h. About 10,000 colonies were selected and used in the biofilm formation assay. Mutants showing 50% up- or downregulated biofilm formation and no significant changes in growth (<20% change) compared to that of the WT were selected in the subsequent experiments.

Mapping the transposon insertion. To map the location of the mini-Tn5 insertion, chromosomal DNA of the mutants was prepared and digested with BamHI or SpeI (all these enzymes do not cut within the transposon mini-Tn5). The resulting fragments were self-ligated and used to transform *E. coli* UQ3021 cells. Self-ligation products harboring mini-Tn5 were maintained as stable plasmids based on the presence of the R6K origin of replication and allowed selection on kanamycin-containing plates. Subsequently, transposon junction plasmids were prepared from selected transformants and the regions flanking the mini-Tn5 were sequenced using primers Tn5-seq1/Tn5-seq2 (Table 2), which anneal to the oriR6K and Km^r ends of the transposon, respectively. The resulting sequences were searched in GenBank to reveal the location of the transposon insertion.

Construction of single, double, and triple mutants. Deletion mutants of the *Sputn32_0303* gene and *Sputn32_0304-0303* operon were constructed by gene replacement based on homologous recombination according to a previous study (56). To construct the Δ0303 mutant, a 1,399-bp upstream region and a 1,420-bp downstream region were amplified using primers 0303-5F/0303-5R and 0303-3F/0303-3R, respectively. The genomic DNA of WT *S. putrefaciens* CN32 was used as a PCR template. These two fragments were cloned into a pUX19 vector at the BamHI and EcoRI sites, yielding plasmid pUX0303UD. Then the *aacC1* gene (encoding Gm^r) from the pUCGm vector was inserted into pUX0303UD at the PstI site, resulting in suicide plasmid pUX0303UD-Gm. Plasmid pUX0303UD-Gm was introduced from *E. coli* DH5α into WT *S. putrefaciens* CN32 using helper plasmid pRK2013 by triparental conjugation, which was performed at 28°C for 8 h. Transconjugants were screened as Gm^r Km^s colonies. Allelic exchanges were confirmed by PCR analysis using primers 0303-UF/0303-UR, 0303-DF/0303-DR, 0303-OF/0303-OR, and GUR/GDF. Using this method, we constructed the Δ0303Δ0304 mutant. Allelic exchanges were confirmed by analysis using primers 0303-UF/0303-UR, 34-DF/34-DR, 0303-OF/0304-OR, and GUR/GDF.

All other deletion mutants were constructed according to previous studies (39–41). To construct the Δ0304 mutant, PCR was used to generate a 1,587-bp DNA fragment upstream (positions –1443 to 144 relative to the *Sputn32_0304* start codon) and a 1,529-bp DNA fragment downstream (positions 565 to 2094 relative to the *Sputn32_0304* start codon) of the *Sputn32_0304* gene (reserving 144 bp after the start codon for real-time RT-PCR analysis) using primers 0304-5F/0304-5R and 0304-3F/0304-3R, respectively. The WT genomic DNA was used as a PCR template. The PCR products of the upstream and downstream regions of the target gene were linked together by fusion PCR. The linked DNA fragment was digested with BamHI and EcoRI and cloned into the suicide vector pK19mobsacB, yielding pK0304UD, which was introduced into WT *S. putrefaciens* CN32 via triparental conjugation. The deletion mutants were obtained by double selection on LB agar supplemented with kanamycin and NaCl-free LB agar containing 10% sucrose. Double-crossover recombination was confirmed by PCR analysis using primers 0304-UF/0304-DR and 0304-OF/0304-OR. Using this method, we constructed the Δ0305, Δ0303Δ0305, Δ0304Δ0305, and Δ0303Δ0304Δ0305 mutants. All the mutants were confirmed by sequencing analysis.

Construction of complementation strains. For complementation of the three single-mutant strains Δ0303, Δ0304, and Δ0305, DNA fragments carrying the ribosome binding site (RBS) and complete ORFs of the *Sputn32_0303*, *Sputn32_0304*, and *Sputn32_0305* genes without their own promoters were amplified using primers 0303-comF/0303-comR, 0304-comF/0304-comR, and 0305-comF/0305-comR, respectively. Then the DNA fragments were cloned into the BamHI and EcoRI sites of pBBR1MCS-2-P_{*aacC1*} (constructed by our laboratory, the promoter of *aacC1* gene was linked to pBBR1MCS-2), thereby yielding the complementation vectors pBBR-P_{*aacC1*}-0303, pBBR-P_{*aacC1*}-0304, and pBBR-P_{*aacC1*}-0305, respectively. The coding regions of *Sputn32_0303*, *Sputn32_0304*, and *Sputn32_0305* with their own RBSs were under the control of an *aacC1* (encoding Gm^r) promoter. The three plasmids were introduced into Δ0303, Δ0304, and Δ0305 to obtain the complementation strains C-0303, C-0304, and C-0305, respectively.

TABLE 2 Primers used in this study

Primer	Sequence (5'–3') ^b	Description, target gene, or purpose
Tn5-seq1	AACAAGCCAGGGATGTAACG	Sequence primer
Tn5-seq2	CAGCAACACCTTCTTCACGA	Sequence primer
0303-5F	AATTTAGAATTCGATAAATGGTCAACATTCTATCCAG	<i>Sputcn32_0303</i>
0303-5R	GTAAATCTGCAGCGCTCTAATGTTTTGGTGATCTAT	<i>Sputcn32_0303</i>
0303-3F	CAATATCTGCAGGATCTTCATATGATGCTGTTTCGC	<i>Sputcn32_0303</i>
0303-3R	CTAGGCGGATCCAAATATCTGTACTTAGCGAGGAAAC	<i>Sputcn32_0303</i>
0303-UF	AACATTCGACATGGACACAAAGCC	<i>Sputcn32_0303</i>
0303-UR	GCAACAAGCGTGGTAAGTGCCTGTG	<i>Sputcn32_0303</i>
0303-DF	TAGAGTGACCATATAGCGTAAGC	<i>Sputcn32_0303</i>
0303-DR	TCGTGAGATGGTCGTCTACAAC	<i>Sputcn32_0303</i>
0303-OF	TTGAGCTATCGAAATTTACCCACC	<i>Sputcn32_0303</i>
0303-OR	GATCATCGAGAAGATTGTGATGG	<i>Sputcn32_0303</i>
GUR	CGAATGATGCCATACTTGAGCC	<i>Sputcn32_0303</i>
GDF	GTTGGGCATACGGGAAGAAGTG	<i>Sputcn32_0303</i>
34-3F	TTTCATCTGCAGTCATGTCGTCCAGAGTTAAGAAG	<i>Sputcn32_0303-0304</i>
34-3R	AAAAAAGGATCCGATCATCGAGAAGATTGTGATGG	<i>Sputcn32_0303-0304</i>
34-DF	CTAGTATGATCTTGAGTGCTAAAC	<i>Sputcn32_0303-0304</i>
34-DR	AATATATACATACGTTACTGCACG	<i>Sputcn32_0303-0304</i>
0304-5F	AACGGTGGATCCTTTCTAATGCCTGTAACATCATCGGG	<i>Sputcn32_0304</i>
0304-5R	CAATTAATCTACTAATGTCTTAGCATTAAAGGGAGATAAATCTTTAACGAGTTTCAGTGC	<i>Sputcn32_0304</i>
0304-3F	GCACTGAAACTCGTTAAAGATTATCTCCCTTAATGCTA AGACATTAGTAGATTTAATTG	<i>Sputcn32_0304</i>
0304-3R	CGCTTGGAAATCAAATCGCAGCTTGACATCTTGTTTC	<i>Sputcn32_0304</i>
0304-UF	CATCCATTGTATATCAAAAGGGC	<i>Sputcn32_0304</i>
0304-DR	ATGTACACAGACACATAGTTCTAC	<i>Sputcn32_0304</i>
0304-OF	TGCATCTAGTCGACGAATGTAGTG	<i>Sputcn32_0304</i>
0304-OR	CCGTTATAGAGATTATTATTAGCC	<i>Sputcn32_0304</i>
345-5F	TTGTGTGAATTCATGCGACCGCGGAAGTATTAATTG	<i>Sputcn32_0303-0304-0305</i>
345-5R	TACTTCCGTTATAGAGATTATTATTAGCCGTAATCGCTCTAATGTTTTGGTGATC	<i>Sputcn32_0303-0304-0305</i>
345-3F	GATCACCAAAAACATTAGAGCGATTTACGGGCTAATAAATCTCTATAACGGAAGTA	<i>Sputcn32_0303-0304-0305</i>
345-3R	GCGGCGCTGCAGCTATTTCTACAGATTTACTCAACCA	<i>Sputcn32_0303-0304-0305</i>
345-UF	CTGCTATGGGATTGGTTATAGC	<i>Sputcn32_0303-0304-0305</i>
345-OF	TGGATGGTATGACCCAATGGGC	<i>Sputcn32_0303-0304-0305</i>
0305-5F	AACTGCAGCTTCATATGATGCTGTTTCGCTTC	<i>Sputcn32_0305</i>
0305-5R	AAACGCTGAATAAATGTTTGTTCATCTATCTCCAATACAGTACGTTGAAAAGGATGATC	<i>Sputcn32_0305</i>
0305-3F	GATCATCCTTTTCAACGTAAGTATTGGAGATAGATGAACAAACATTTATTCAGCGTTT	<i>Sputcn32_0305</i>
0305-3R	CATTGCGAATTCCTAGCCAATCATATTGCCAATGCC	<i>Sputcn32_0305</i>
0305-UF	AATAGTGGGTTACCACAATTTG	<i>Sputcn32_0305</i>
0305-DR	AGTCAGATATATCCTGATTAGTG	<i>Sputcn32_0305</i>
0305-OF	GATACCAACACGATCTTACCATG	<i>Sputcn32_0305</i>
0305-OR	CTTATCAAGCTACTGAGCGACG	<i>Sputcn32_0305</i>
45-5F	ACTGTACTGCAGAAGGCTAATAACTCTAGTACATCCG	<i>Sputcn32_0304-0305</i>
45-3R	CGGCGGGAATTCCGGCGCTATTTCTACAGATTTACTC	<i>Sputcn32_0304-0305</i>
45-OF	ACTGTGAGATACTACGGCAAAGGG	<i>Sputcn32_0304-0305</i>
0303-comF	TTAATTGGATCCGAAATAGCGAAGCGAAACAGCATC	<i>Sputcn32_0303</i>
0303-comR	CCCCTGGGTACCATAGATCACCAAAAACATTAGAGC	<i>Sputcn32_0303</i>
0304-comF	ATAACAGGATCCTACAAACGGTTAGAATGTGTATC	<i>Sputcn32_0304</i>
0304-comR	CAATATGAATTCCTCACTTCATATGATGCTGTTTCGC	<i>Sputcn32_0304</i>
0305-comF	TTTGTAGGATCCATGGAGCAAGTAAAAAGCATGTG	<i>Sputcn32_0305</i>
0305-comR	ACAATTGAATTCATGCTGCAATATGGTCATTATGTC	<i>Sputcn32_0305</i>
0304D54A-F ^a	GATGCAGTTATTCTAGCAATAGGTATTCCT	<i>Sputcn32_0304 D54A</i>
0304D54E-F ^a	GATGCAGTTATTCTAGAGATAGGTATTCCT	<i>Sputcn32_0304 D54E</i>
0304D54-R ^a	GGGAGATAAATCTTTAACGAGTTTCAGTGC	<i>Sputcn32_0304 D54A/E</i>
0305D56A-F ^a	GACATCGTCATATGTGCATTGATGATGACG	<i>Sputcn32_0305 D56A</i>
0305D56E-F ^a	GACATCGTCATATGTGAGTTGATGATGACG	<i>Sputcn32_0305 D56E</i>
0305D56-R ^a	CGCACTATGGTGAATATCGAGCCACGCTAG	<i>Sputcn32_0305 D56A/E</i>
0305E179A-F ^a	GTTTAAGAGGGGCTGCTGTGCTTGCTCGTT	<i>Sputcn32_0305 E179A</i>
0305E179A-R ^a	TAAACTGAGATACATCAAACCTTTGGTTGAT	<i>Sputcn32_0305 E179A</i>
0303-HPT-F	GCTCTAGAGCAAAGCTAGAAAGCTACTGTCAAAGC	Bacterial two hybrid
0303-HPT-R	GGGGTACCAGAGCGATTTACGCTGCTAAGTG	Bacterial two hybrid
0304-D2-F	CGGGATCCAAACGACATGAATGAAAGAAAAA	Bacterial two hybrid
0304-D2-R	CGGAATTCCTCAAAAATTGGAAGTAGGAATGGCCT	Bacterial two hybrid
0305-D2-F	CGGGATCCAAAGGTATTTATGGGTAAAGATTA	Bacterial two hybrid
0305-D2-R	CGGAATTCCTCAAGGAACTTTTTATTTACTTTG	Bacterial two hybrid
RocS2-HPT-F	GCTCTAGAGGCGACCTTTTCGGACGCGA	Bacterial two hybrid

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5'–3') ^b	Description, target gene, or purpose
RocS2-HPT-R	GGGGTACCGGAAGGGCTCATACGCAGC	Bacterial two hybrid
RocA2-D2-F	CGGGATCCAAATCAGACAATGAGCCGTA	Bacterial two hybrid
RocA2-D2-R	CGGAATTCCTCAAACCGAGCTGGGAAAGTAACTG	Bacterial two hybrid
0304P-F	ACATGAGCTAGCATGAAAAGAAAATATTGATAGTGG	EMSA protein
0304P-R	TGATCTGGATCCTCATATGATGCTGTTTCGCTTCGC	EMSA protein
0304_0305_int-F	TCATGTCGTTCCAGAGTTAAGAAG	EMSA probe
0304_0305_int-R	AAAATACCTTTAAGAAATCAATAAAAC	EMSA probe
P1-R	ACTTACAACCTTAGATGTACACAG	EMSA probe
P2-F	AGTTTTACGTGCAGTAACGTATGT	EMSA probe
P3-R	AGTTCTACTATTTTATGATCATTC	EMSA probe
P4-F	ACTATGTGTCTGTGTACATCTAA A	EMSA probe
P5-R	ATTCTAAAACCTGAAATGGCTGAAT	EMSA probe
P6-F	AATGATCATAAAATAGTAGAACTA	EMSA probe
0303-QF	TACATCCGCGGCAAACTCTT	Real-time RT-PCR
0303-QR	TGGAATGGTGGCGGGATAAC	Real-time RT-PCR
0304-QF	GGAGATAAATCTTTAACGAG	Real-time RT-PCR
0304-QR	ATTGATAGTGGATGATCACC	Real-time RT-PCR
0305-QF	CCTTTGGTACGGCACAGGAT	Real-time RT-PCR
0305-QR	ATCATCTGGGAAACCGAGCG	Real-time RT-PCR
recA-QF	ATAACGTCAACAGCGCCTGA	Real-time RT-PCR
recA-QR	CGCAGCGTGAAGGTAAAAC	Real-time RT-PCR
Oligo(dT) anchor-primer	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTT	5' RACE
SP1-0304	TGGCCTGATAATATCGCTCGAAG	5' RACE
SP2-0304	CAGTGCATCAACTCCATTATTAG	5' RACE
SP1-0305	TACTTTGCGATCTGAGAATTTATC	5' RACE
SP2-0305	AAGGTTATACTCCAATACAGTACG	5' RACE

^a5' phosphorylated primer.^bUnderlining indicates the restriction enzyme site.**Construction of *Sputcn32_0304* and *Sputcn32_0305* mutants via site-directed mutagenesis.**

The phosphorylation sites of *Sputcn32_0304* and *Sputcn32_0305* were predicted by the NCBI website (<https://www.ncbi.nlm.nih.gov>). The catalytic motif of PDE activity in *Sputcn32_0305* was obtained by blasting with RocR. The *Sputcn32_0304* and *Sputcn32_0305* site-directed mutants C-0304-D54A, C-0304-D54E, C-0305-D56A, C-0305-D56E, and C-0305-E179A were constructed by site-directed mutagenesis. Mutant plasmids pBBR-P_{aacC1}-0304-D54A, pBBR-P_{aacC1}-0304-D54E, pBBR-P_{aacC1}-0305-D56A, pBBR-P_{aacC1}-0305-D56E, and pBBR-P_{aacC1}-0305-E179A were constructed on the complementation plasmids pBBR-P_{aacC1}-0304 and pBBR-P_{aacC1}-0305 by using the 5'-phosphorylated mutagenic primers 0304D54A-F, 0304D54E-F, 0304D54-R, 0305D56A-F, 0305D56E-F, 0305D56-R, 0305E179A-F, and 0305E179A-R. The plasmids with the mutant alleles were introduced into Δ0304 and Δ0305.

Biofilm formation plate assay and assay of different carbon sources. The static biofilm formation assay was based on a 96-well plate method as described elsewhere (46). Biofilm formation was assessed using a gradient concentration of different carbon sources. The concentration of sodium lactate in MM1 medium was adjusted to 5 mM, 10 mM, 20 mM, and 30 mM. For the sodium acetate or sodium propionate concentration gradient assay, 10 mM, 20 mM, and 30 mM sodium acetate or sodium propionate were added to MM1 medium without sodium lactate, respectively. Yeast extract at 1.7 g/liter was added to MM1 medium to replace sodium lactate as the sole carbon source in a control medium.

Bacterial two-hybrid experiments. Bacterial two-hybrid experiments were conducted as described elsewhere (38). We constructed pKT25-0303HPT, pUT18C-0304D2, and pUT18C-0305D2. The positive control of the bacterial two-hybrid experiment was a known TCS, RocS2-RocA2, in *Pseudomonas aeruginosa* PAO1 (38). The negative controls were BTH101 with pKT25 and pUT18C, as well as BTH101 with pKT25-0303HPT and pUT18C-RocA2.

Flow cell biofilm. The hydrodynamic biofilm formation assay was performed as described elsewhere, with slight modifications (46). Biofilms were cultivated at 28°C in three-channel flow cells; the flow cell system was set up according to previous descriptions (46, 47). The WT and mutant bacterial strains were cultured overnight in LB medium and adjusted to an optical density at 600 nm (OD₆₀₀) of 0.5. The diluted cells were injected into each channel. The chambers were turned upside down for 2 h to promote initial attachment. Then the flow chambers were inverted, and the MM1 medium was continuously supplied at a constant rate of 12 ml/h per channel by using a peristaltic pump (Ismatec, Germany). Biofilm formation in the flow cell system after 48 h was observed by using a confocal laser scanning microscope (CLSM) (Olympus FV1000). Images were analyzed using Imaris software.

Real-time RT-PCR analysis. WT and derivative strains were grown at 28°C. The cells were cultured in MM1 medium and collected at 6 h. Total cellular RNA was isolated using an SV total RNA isolation system (Promega). cDNA was then synthesized by using a reverse transcription system (Promega). The real-time RT-PCR system and amplification parameters were set up according to the protocol of the Power SYBR green PCR master mix (Applied Biosystems). Signal intensities of the PCR products were

standardized to that of the *recA* gene (57). The data were analyzed as described elsewhere (57, 58). Each experiment was performed in triplicate.

Protein purification. The coding region of *Sputn32_0304* was obtained by PCR using primers 0304P-F/0304P-R. The PCR product was cut with *NheI*/*Bam*HI, and then cloned into pET-28a (+) to generate the expression plasmid pET28-0304. The site-directed plasmids were constructed with this plasmid by using 5'-phosphorylated mutagenic primers. The resulting plasmids were confirmed by DNA sequencing and then transformed into *E. coli* BL21(DE3) for overexpression of His₆-0304, His₆-0304D54E, and His₆-0304D54A (six-histidine tag on the N termini of proteins). Following induction with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 16°C for 24 h, the recombinant proteins were purified by Ni-nitrilotriacetic acid agarose chromatography (GE Healthcare) according to the manufacturer's protocol.

Electrophoretic mobility shift assay (EMSA). A digoxigenin (DIG) gel shift kit (second generation; Roche) was used. DNA probes 0304_0305_int were PCR amplified using the primers listed in Table 2 and labeled with DIG at the 3'-terminal ends. The reaction mixture (20 μ l) contained the proteins, DNA probes, and 1 μ g of poly(dA-dT) (vial 10) in the binding buffer (vial 5). Following incubation at 25°C for 30 min, the binding reactions were separated by electrophoresis (with 5% native polyacrylamide gels and 0.5 $\times$ Tris-borate-EDTA [TBE] as the running buffer). Then the following procedures were carried out according to a previous study (39). A 12.5 mM concentration of acetyl phosphate (AcP; Sigma) or phosphorylation buffer (50 mM Tris-HCl [pH 7.6], 50 mM KCl, 20 mM MgCl₂) was incubated with the recombinant His₆-0304 at 25°C for 60 min and 120 min *in vitro* according to procedures in a previous study (43).

Determination of transcriptional start sites (TSSs) by 5' RACE. The 5' RACE experiment was conducted as described elsewhere (39).

c-di-GMP extraction and quantification. The WT and single-mutant strains were grown in 150-ml flasks containing 50 ml of MM1 medium at 28°C with shaking at 200 rpm and cultured for 6 h. The c-di-GMP extraction and quantification were conducted as described elsewhere (59). Total proteins were quantified with a Bradford protein assay reagent (Genstar). The c-di-GMP was analyzed by using an Agilent Technologies 6400 Triple Quad LC/MS. Separation was achieved on a reverse-phase C₁₈ column. The column was set at 25°C, with a flow rate of 1 ml/min. The samples were separated with a 22.01-min gradient: min 0 to 6, 10% B; min 6 to 10, 80% B; and min 10 to 22, 10% B (A, 10 mM ammonium acetate; B, methanol). The maximum pressure limit was 4 $\times$ 10⁷ Pa. c-di-GMP (Biolog Life Science Institute, Germany) in double-distilled water (ddH₂O) was used as a standard.

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REFERENCES

1. Vert M, Doi Y, Hellwich KH, Hess M, Hodge P, Kubisa P, Rinaudo M, Schué F. 2012. Terminology for biorelated polymers and applications (IUPAC recommendations 2012). *Pure Appl Chem* 84:377–410. <https://doi.org/10.1351/PAC-REC-10-12-04>.
2. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. 2016. Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 14:563–575. <https://doi.org/10.1038/nrmicro.2016.94>.
3. Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2:95–108. <https://doi.org/10.1038/nrmicro821>.
4. Camilli R, Pantosti A, Baldassarri L. 2011. Contribution of serotype and genetic background to biofilm formation by *Streptococcus pneumoniae*. *Eur J Clin Microbiol Infect Dis* 30:97–102. <https://doi.org/10.1007/s10096-010-1060-6>.
5. Kim J, Pitts B, Stewart PS, Camper A, Yoon J. 2008. Comparison of the antimicrobial effects of chlorine, silver ion, and tobramycin on biofilm. *Antimicrob Agents Chemother* 52:1446–1453. <https://doi.org/10.1128/AAC.00054-07>.
6. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436:1171–1175. <https://doi.org/10.1038/nature03912>.
7. Kaplan JB, Izano EA, Gopal P, Karwacki MT, Kim S, Bose JL, Bayles KW, Horswill AR. 2012. Low levels of β -lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. *mBio* 3:e00198–12. <https://doi.org/10.1128/mBio.00198-12>.
8. Ng M, Epstein SB, Callahan MT, Piotrowski BO, Simon GL, Roberts AD, Keiser JF, Kaplan JB. 2014. Induction of MRSA biofilm by low-dose β -lactam antibiotics: specificity, prevalence and dose-response effects. *Dose Response* 12:152–161. <https://doi.org/10.2203/dose-response.13-021.Kaplan>.
9. Capra EJ, Laub MT. 2012. Evolution of two-component signal transduction systems. *Annu Rev Microbiol* 66:6511–6517. <https://doi.org/10.1146/annurev-micro-092611-150039>.
10. Guckes KR, Kostakioti M, Breland EJ, Gu AP, Shaffer CL, Rd MC, Hultgren SJ, Hadjifrangiskou M. 2013. Strong cross-system interactions drive the activation of the QseB response regulator in the absence of its cognate sensor. *Proc Natl Acad Sci U S A* 110:16592–16597. <https://doi.org/10.1073/pnas.1315320110>.
11. Alm E, Huang K, Arkin A. 2006. The evolution of two-component systems in bacteria reveals different strategies for niche adaptation. *PLoS Comput Biol* 2:1329–1342. <https://doi.org/10.1371/journal.pcbi.0020143>.
12. Ashenberg O, Rozen-Gagnon K, Laub MT, Keating AE. 2011. Determinants of homodimerization specificity in histidine kinases. *J Mol Biol* 413:222–235. <https://doi.org/10.1016/j.jmb.2011.08.011>.
13. Chambonnier G, Roux L, Redelberger D, Fadel F, Filloux A, Sivaneson M, Bentzmann SD, Bordin C. 2016. The hybrid histidine kinase LadS forms a multicomponent signal transduction system with the GacS/GacA two-component system in *Pseudomonas aeruginosa*. *Plos Genet* 12:e1006032. <https://doi.org/10.1371/journal.pgen.1006032>.
14. Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S. 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* 73:434–445. <https://doi.org/10.1111/j.1365-2958.2009.06782.x>.
15. Sall KM, Casabona MG, Bordin C, Huber P, de Bentzmann S, Attrée I, Elsen S. 2014. A *gacS* deletion in *Pseudomonas aeruginosa* cystic fibrosis isolate CHA shapes its virulence. *PLoS One* 9:e95936. <https://doi.org/10.1371/journal.pone.0095936>.
16. Brosse A, Korobeinikova A, Gottesman S, Guillier M. 2016. Unexpected

- properties of sRNA promoters allow feedback control via regulation of a two-component system. *Nucleic Acids Res* 44:9650–9666.
17. Kulasekara HD, Ventre I, Kulasekara BR, Lazdunski A, Filloux A, Lory S. 2005. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. *Mol Microbiol* 55:368–380. <https://doi.org/10.1111/j.1365-2958.2004.04402.x>.
 18. Meissner A, Wild V, Simm R, Rohde M, Erck C, Bredenbruch F, Morr M, Römling U, Häussler S. 2007. *Pseudomonas aeruginosa* *cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. *Environ Microbiol* 9:2475–2485. <https://doi.org/10.1111/j.1462-2920.2007.01366.x>.
 19. Kuchma SL, Connolly JP, O'Toole GA. 2005. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* 187:1441–1454. <https://doi.org/10.1128/JB.187.4.1441-1454.2005>.
 20. Sondermann H, Shikuma NJ, Yildiz FH. 2012. You've come a long way: c-di-GMP signaling. *Curr Opin Microbiol* 15:140–146. <https://doi.org/10.1016/j.mib.2011.12.008>.
 21. Opoku-Temeng C, Jie Z, Yue Z, Su J, Sintim HO. 2016. Cyclic dinucleotide (c-di-GMP, c-di-AMP, and cGAMP) signalings have come of age to be inhibited by small molecules. *Chem Commun (Camb)* 52:9327–9342. <https://doi.org/10.1039/C6CC03439J>.
 22. Purcell EB, Tamayo R. 2016. Cyclic diguanylate signaling in Gram-positive bacteria. *FEMS Microbiol Rev* 40:753–773. <https://doi.org/10.1093/femsre/fuw013>.
 23. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7:263–273. <https://doi.org/10.1038/nrmicro2109>.
 24. Ha DG, O'Toole GA. 2015. c-di-GMP and its effects on biofilm formation and dispersion: a *Pseudomonas aeruginosa* review. *Microbiol Spectr* 3:MB-0003-2014. <https://doi.org/10.1128/microbiolspec.MB-0003-2014>.
 25. Boehm A, Kaiser M, Li H, Spangler C, Kasper C, Ackermann M, Kaever V, Sourjik V, Roth V, Jenal U. 2010. Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* 141:107–116. <https://doi.org/10.1016/j.cell.2010.01.018>.
 26. Paul R, Weiser S, Amiot NC, Chan C, Schirmer T, Giese B, Jenal U. 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev* 18:715–727. <https://doi.org/10.1101/gad.289504>.
 27. Galperin MY. 2004. Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* 6:552–567. <https://doi.org/10.1111/j.1462-2920.2004.00633.x>.
 28. Wu C, Cheng YY, Yin H, Song XN, Li WW, Zhou XX, Zhao LP, Tian LJ, Han JC, Yu HQ. 2013. Oxygen promotes biofilm formation of *Shewanella putrefaciens* CN32 through a diguanylate cyclase and an adhesin. *Sci Rep* 3:1945. <https://doi.org/10.1038/srep01945>.
 29. Khalid A, Arshad M, Crowley DE. 2008. Decolorization of azo dyes by *Shewanella* sp. under saline conditions. *Appl Microbiol Biotechnol* 79:1053–1059. <https://doi.org/10.1007/s00253-008-1498-y>.
 30. Kozłńska A, Pékala A. 2004. First isolation of *Shewanella putrefaciens* from freshwater fish—a potential new pathogen of the fish. *Bull Eur Assoc Fish Pathol* 24:189–193.
 31. Lei Q, Zhang X. 2012. A new pathogen of gibel carp *Carassius auratus* gibelio-*Shewanella putrefaciens*. *Acta Microbiol Sin* 52:558–565.
 32. Pékala A, Kozłńska A, Paździor E, Glowacka H. 2015. Phenotypical and genotypical characterization of *Shewanella putrefaciens* strains isolated from diseased freshwater fish. *J Fish Dis* 38:283–293. <https://doi.org/10.1111/jfd.12231>.
 33. Dhawan B, Chaudhry R, Mishra BM, Agarwal R. 1998. Isolation of *Shewanella putrefaciens* from a rheumatic heart disease patient with infective endocarditis. *J Clin Microbiol* 36:2394–2394.
 34. Pagani L, Lang A, Vedovelli C, Moling O, Rimenti G, Pristerà R, Mian P. 2003. Soft tissue infection and bacteremia caused by *Shewanella putrefaciens*. *J Clin Microbiol* 41:2240–2241. <https://doi.org/10.1128/JCM.41.5.2240-2241.2003>.
 35. Zhou G, Yuan J, Gao H. 2015. Regulation of biofilm formation by BpfA, BpfD, and BpfG in *Shewanella oneidensis*. *Front Microbiol* 6:790. <https://doi.org/10.3389/fmicb.2015.00790>.
 36. Theunissen S, De Smet L, Dansercoer A, Motte B, Coenye T, Van Beeumen JJ, Devreese B, Savvides SN, Vergauwen B. 2010. 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. <https://doi.org/10.1016/j.resmic.2009.12.002>.
 37. Cheng YY, Wu C, Wu JY, Jia HL, Wang MY, Wang HY, Zou SM, Sun RR, Jia R, Xiao YZ. 2016. FlrA represses transcription of biofilm-associated *bpfA* operon in *Shewanella putrefaciens*. *Appl Environ Microbiol* <https://doi.org/10.1128/AEM.02410-16>.
 38. Sivaneson M, Mikkelsen H, Ventre I, Bordi C, Filloux A. 2011. Two-component regulatory systems in *Pseudomonas aeruginosa*: an intricate network mediating fimbrial and efflux pump gene expression. *Mol Microbiol* 79:1353–1366. <https://doi.org/10.1111/j.1365-2958.2010.07527.x>.
 39. Liu W, Zhang Q, Guo J, Chen Z, Li J, Wen Y. 2015. Increasing avermectin production in *Streptomyces avermitilis* by manipulating the expression of a novel TetR-family regulator and its target gene product. *Appl Environ Microbiol* 81:5157–5173. <https://doi.org/10.1128/AEM.00868-15>.
 40. Sun D, Zhu J, Chen Z, Li J, Wen Y. 2016. SAV742, a novel *araC*-family regulator from *Streptomyces avermitilis*, controls avermectin biosynthesis, cell growth and development. *Sci Rep* 6:36915. <https://doi.org/10.1038/srep36915>.
 41. Zhu J, Sun D, Liu W, Chen Z, Li J, Wen Y. 2016. AvaR2, a pseudo γ -butyrolactone receptor homologue from *Streptomyces avermitilis*, is a pleiotropic repressor of avermectin and avenolide biosynthesis and cell growth. *Mol Microbiol* 102:562–578. <https://doi.org/10.1111/mmi.13479>.
 42. Kedzierska B, Lian LY, Hayes F. 2007. Toxin-antitoxin regulation: bimodal interaction of YefM-YoeB with paired DNA palindromes exerts transcriptional autorepression. *Nucleic Acids Res* 35:325–339. <https://doi.org/10.1093/nar/gkl1028>.
 43. He H, Zahrt TC. 2005. Identification and characterization of a regulatory sequence recognized by *Mycobacterium tuberculosis* persistence regulator MprA. *J Bacteriol* 187:202–212. <https://doi.org/10.1128/JB.187.1.202-212.2005>.
 44. Tzeng YL, Zhou X, Bao S, Zhao S, Noble C, Stephens DS. 2006. Autoregulation of the MisR/MisS two-component signal transduction system in *Neisseria meningitidis*. *J Bacteriol* 188:5055–5065. <https://doi.org/10.1128/JB.188.22.5055-5065>.
 45. Kaspar S, Bott M. 2002. The sensor kinase CitA (DpiB) of *Escherichia coli* functions as a high-affinity citrate receptor. *Arch Microbiol* 177:313–321. <https://doi.org/10.1007/s00203-001-0393-z>.
 46. Ding Y, Peng N, Du Y, Ji L, Cao B. 2014. Disruption of putrescine biosynthesis in *Shewanella oneidensis* enhances biofilm cohesiveness and performance in Cr(VI) immobilization. *Appl Environ Microbiol* 80:1498–1506. <https://doi.org/10.1128/AEM.03461-13>.
 47. Thormann KM, Saville RM, Shukla S, Pelletier DA, Spormann AM. 2004. Initial phases of biofilm formation in *Shewanella oneidensis* MR-1. *J Bacteriol* 186:8096–8104. <https://doi.org/10.1128/JB.186.23.8096-8104.2004>.
 48. Zhang W, Seminara A, Suaris M, Brenner MP, Weitz DA, Angelini TE. 2014. Nutrient depletion in *Bacillus subtilis* biofilms triggers matrix production. *New J Phys* 16:015028. <https://doi.org/10.1088/1367-2630/16/1/015028>.
 49. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* 48:1511–1524. <https://doi.org/10.1046/j.1365-2958.2003.03525.x>.
 50. Aboulkacem A, Barguigua A, Maroui I, Timinouni M, Belhaj A. 2014. Influence of both media carbon source and strains variety on the biofilms formation ability by *Pseudomonas aeruginosa*. *Int J Innov Sci Res* 12:190–198.
 51. Heydorn A, Ersbøll B, Kato J, Hentzer M, Parsek MR, Tolker-Nielsen T, Givskov M, Molin S. 2002. Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. *Appl Environ Microbiol* 68:2008–2017. <https://doi.org/10.1128/AEM.68.4.2008-2017.2002>.
 52. Römling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77:1–52. <https://doi.org/10.1128/MMBR.00043-12>.
 53. Thormann KM, Duttler S, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM. 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J Bacteriol* 188:2681–2691. <https://doi.org/10.1128/JB.188.7.2681-2691.2006>.
 54. Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, Howell PL, Wozniak DJ, Parsek MR. 2012. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ Microbiol* 14:1913–1928. <https://doi.org/10.1111/j.1462-2920.2011.02657.x>.
 55. Larsen RA, Wilson MM, Guss AM, Metcalf WW. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a

- wide variety of bacteria. *Arch Microbiol* 178:193–201. <https://doi.org/10.1007/s00203-002-0442-2>.
56. Ding Y, Li J, Liu J, Yang J, Jiang W, Tian J, Li Y, Pan Y, Li J. 2010. Deletion of the *ftsZ*-like gene results in the production of superparamagnetic magnetite magnetosomes in *Magnetospirillum gryphiswaldense*. *J Bacteriol* 192:1097–1105. <https://doi.org/10.1128/JB.01292-09>.
 57. Gralnick JA, Brown CT, Newman DK. 2005. Anaerobic regulation by an atypical Arc system in *Shewanella oneidensis*. *Mol Microbiol* 56:1347–1357. <https://doi.org/10.1111/j.1365-2958.2005.04628.x>.
 58. Mohanty A, Kathawala MH, Zhang J, Chen WN, Loo JSC, Kjelleberg S, Yang L, Cao B. 2014. Biogenic tellurium nanorods as a novel antiviral agent inhibiting pyoverdine production in *Pseudomonas aeruginosa*. *Biotechnol Bioeng* 111:858–865. <https://doi.org/10.1002/bit.25147>.
 59. Zhu B, Liu C, Liu S, Cong H, Chen Y, Gu L, Ma LZ. 2016. Membrane association of SadC enhances its diguanylate cyclase activity to control exopolysaccharides synthesis and biofilm formation in *Pseudomonas aeruginosa*. *Environ Microbiol* 18:3440–3452. <https://doi.org/10.1111/1462-2920.13263>.
 60. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotechnol* 1:784–791. <https://doi.org/10.1038/nbt1183-784>.
 61. Karimova G, Ladant D. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A* 95:5752–5756. <https://doi.org/10.1073/pnas.95.10.5752>.
 62. Zhang Y, Pohlmann EL, Roberts GP. 2005. GlnD is essential for NifA activation, NtrB/NtrC-regulated gene expression, and posttranslational regulation of nitrogenase activity in the photosynthetic, nitrogen-fixing bacterium *rhodospirillum rubrum*. *J Bacteriol* 187:1254–1265. <https://doi.org/10.1128/JB.187.4.1254-1265.2005>.
 63. Wohlleben W, Arnold W, Bissonnette L, Pelletier A, Tanguay A, Roy PH, Gamboa GC, Barry GF, Aubert E, Davies J. 1989. On the evolution of Tn21-like multiresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I(AAC(3)-I), another member of the Tn21-based expression cassette. *Mol Genet Genomics* 217:202–208. <https://doi.org/10.1007/BF02464882>.
 64. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145:69–73. [https://doi.org/10.1016/0378-1119\(94\)90324-7](https://doi.org/10.1016/0378-1119(94)90324-7).
 65. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Li RMR, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176. [https://doi.org/10.1016/0378-1119\(95\)00584-1](https://doi.org/10.1016/0378-1119(95)00584-1).
 66. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* 76:1648–1652. <https://doi.org/10.1073/pnas.76.4.1648>.
 67. Lamberts L, Sternberg C, Molin S. 2004. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* 6:726–732. <https://doi.org/10.1111/j.1462-2920.2004.00605.x>.