### Symbiosis of a P2-Family Phage and Deep-Sea Shewanella putrefaciens

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Running head: P2-family prophage in Shewanella

### **Summary**

Almost all bacterial genomes harbor prophages yet it remains unknown why prophages integrate into tRNA-related genes. Approximately 1/3 of *Shewanella* isolates harbor a prophage at the tmRNA (*ssrA*) gene. Here, we discovered a P2-family prophage integrated at the 3'-end of *ssrA* in the deep-sea bacterium *S. putrefaciens*. We found that ~0.1% of host cells are lysed to release P2 constitutively during host growth. P2 phage production is induced by a prophage-encoded Rep protein and its excision is induced by the Cox protein. We also found that P2 genome excision leads to the disruption of wobble base pairing of SsrA due to site-specific recombination, thus disrupting the trans-translation function of SsrA. We further demonstrated that P2 excision greatly hinders growth in sea water medium and inhibits biofilm formation. Complementation with a functional SsrA in the P2-excised strain completely restores the growth defects in sea water medium and partially restores biofilm formation. Additionally, we found that products of the P2 genes also increase biofilm formation. Taken together, this study illustrates a symbiotic relationship between P2 and its marine host, thus providing multiple benefits for both sides when a phage is integrated but suffers from reduced fitness when the prophage is excised.

### Introduction

Bacteriophages (phages) are the most abundant life entities and are considered to be one of the major drivers of ecological and biogeochemical cycling in the ocean (Paul *et al.*, 2002; Suttle, 2007; Rohwer and Thurber, 2009; Roux *et al.*, 2016). Phages can structure microbial communities, promote bacterial genome evolution and serve as vehicles for gene transfer upon infection (Suttle, 2007; Breitbart, 2012; Alivisatos *et al.*, 2015; Howard-Varona *et al.*, 2017).

Lytic phages are usually considered parasites; however, we have recently shown that the lytic phage SW1 may be used for self-recognition and confers a fitness advantage to kin by lysing predominantly uninfected cells (Song *et al.*, 2019). Temperate phages such as lambda can integrate into the host genome as a prophage or lyse the host upon infection (Little, 1984; Herman *et al.*, 1997; Oppenheim *et al.*, 2005). Once integrated into the host, a temperate phage and its bacterial host adopt strategies to balance the costs and benefits of both parties (Chen *et al.*, 2005; Erez *et al.*, 2017). The integration of phages can broaden a host's own physiological repertoire, including preventing further phage invasion, increasing resistance to serum, and providing multiple benefits to the bacterial host for surviving in adverse environmental conditions (Chen *et al.*, 2005; Wang *et al.*, 2010; Wang and Wood, 2016). In these cases, the reproductive success of the lysogens carrying these new genes can translate directly into evolutionary success for the prophage residing in the chromosome (Canchaya *et al.*, 2003; Varani *et al.*, 2008; Knowles *et al.*, 2016). The phage can either retain its ability to form active phages or degenerate into cryptic phages that are unable to form active phage particles. In the former case, active prophages benefit via passage to bacterial progeny during chromosome replication and exit by lysing the bacterial host when the environment is no longer favorable. In the latter case, cryptic prophages become domesticated, and the fate of the cryptic prophage is tied to its host as they replicate or die together. The establishment of lysogeny for active and cryptic prophages is a fundamental evolutionary question. As André Lwoff once noted, "Lysogeny, a concept once out of favor, is the basis of our understanding of the relations between cell and virus" (Nobel Lecture, December 11, 1963).

Approximately half of bacterial genomes in ecological samples, including those from marine environments, harbor at least one prophage (Paul, 2008; Touchon *et al.*, 2016). DNA-damaging agents such as mitomycin C (MMC) and UV radiation are commonly used to identify active prophages, such as those of the lambda family (Sozhamannan *et al.*, 2006; Asakura *et al.*, 2007; Asadulghani *et al.*, 2009); however, not all prophages are inducible by MMC or UV radiation. Indeed, attempts to identify prophages using MMC or UV radiation have shown very low frequencies of prophage induction in many bacteria (Cochran and Paul, 1998; Knowles *et al.*, 2017), indicating a high frequency of noninducible prophages in host genomes.

P2 is a temperate tailed phage that encodes its own structural proteins and forms active phage particles (Bertani, 1951; Bertani and Bertani, 1970). The first P2 phage was isolated from *E. coli* in 1951, and P2-family prophages are commonly found in  $\gamma$ -proteobacteria (Casjens and Grose, 2016; Christie and Calendar, 2016). In contrast to lambda, P2 has been regarded as the prototype for the noninducible class of temperate phages (Bertani and Bertani, 1970; Liu and Haggard-Ljungquist, 1999). Spontaneous prophage induction (SPI) was first reported by André Lwoff in the 1950s, as cultures of *Bacillus megaterium* lysogens released free phages in the supernatant under noninducing conditions (Lwoff, 1953). SPI is often accompanied by the lysis of only a small population of bacterial cells (Nanda *et al.*, 2015). The occurrence of SPI in different bacteria may represent a complex interaction between prophages and their hosts in group behaviors, such as during biofilm development; however, the significance of SPI and the interplay between the action of SPI and its bacterial host remain largely unexplored.

Metagenomic studies of deep-sea viromes from the Pacific Ocean (The Pacific Ocean Virome data set), the Atlantic Ocean, and the Mediterranean Sea have revealed a high level of diversity and abundance of deep-sea

viruses (Hurwitz and Sullivan, 2013; Winter *et al.*, 2014; Lara *et al.*, 2017). Moreover, the occurrence of lysogeny in the oceans has been found to correlate well with conditions that are unfavorable for rapid host growth (Paul, 2008). For example, lysogeny is more prevalent at mesopelagic depths than in surface waters in the Pacific Ocean (Luo *et al.*, 2017), and lysogeny is the preferred mode for phages in energy-limited deep-sea environments (Mizuno *et al.*, 2016). However, the relationship between marine phages and bacterial hosts in deep-sea ecosystems is still poorly known. P2-family prophages are commonly found in marine  $\gamma$ -proteobacteria as previously described (Casjens and Grose, 2016). Using previously reported metatranscriptome data (Duarte, 2015), we found that active P2 phages are also present in deep-sea microbial communities. Despite the wide distribution of P2 phages and prophages, the molecular understanding of the bacteria-phage metabolic interactions in the deep sea is largely lacking. Thus, the aim of this study was to explore the bacteria-phage relationship for P2 prophage and its host in the deep sea.

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Strains of *Shewanella* have been isolated from various marine habitats, from the ocean surface to the deep ocean floor, and are believed to have a marine origin (Hau and Gralnick, 2007; Fredrickson *et al.*, 2008). In this study, we found nine *Shewanella* strains isolated from diverse marine habitats harboring P2-family prophages, and seven of them are inserted into the *ssrA* gene (small stable RNA A) including the deep-sea strain *S. putrefaciens* W3-18-1 (hereafter W3-18-1). *S. putrefaciens* is a facultative anaerobic psychrophile with relevance to fish spoilage, oil pipeline corrosion, and human infections (Vignier *et al.*, 2013). W3-18-1 was isolated from sediment of the Pacific Ocean (off the coast of Washington State, 667 m in depth) and is particularly interesting because it produces high current in microbial fuel cells and forms magnetite at temperatures as low as 0 °C (Qiu *et al.*, 2013). Thus, a representative P2 prophage in W3-18-1, P2Sp, was selected for studying the P2-host relationship in the deep-sea ecosystem.

Prophage integration bias for tmRNA- or tRNA-related genes is a common phenomenon in different bacteria.

It has been proposed that the use of attachment sites at the 3'-end of tRNAs or tmRNAs ensures the evolutionary stability of the prophage (Williams, 2003; Bobay *et al.*, 2013). The tmRNA encoded by the *ssrA* gene is ubiquitous in bacteria with dual tRNA-like and messenger RNA-like properties and forms a complex with the SsrA-binding protein SmpB to monitor protein synthesis in the cell (Chauhan and Apirion, 1989; Keiler *et al.*, 1996; Himeno *et al.*, 2015). Specifically, SsrA adds tagged peptides to truncated polypeptides for proteolysis using the tRNA-like domain at its 3'-end to ensure rapid degradation of potentially deleterious proteins (Karzai *et al.*, 2000). Recently, we reported that the excision of a P4-like prophage integrated at the 3'-end of *ssrA* in *S. oneidensis* MR-1 is induced at cold temperatures (Zeng *et al.*, 2016). This cold-induced excision inactivates *ssrA* due to site-specific recombination and increases biofilm formation which increases host fitness at low temperatures (Zeng *et al.*, 2016). Hence, excision of the phage is beneficial for growth at low temperatures. Our previous P4 study illustrated how the bacterial host turns a cryptic prophage into a regulatory switch to regulate host behavior without the formation of active phage particles.

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In contrast to P4-family cryptic prophages that do not form active phage particles after excision, all P2-family prophages contain intact phage structural genes. In this study, we discovered a symbiotic relationship between a P2 prophage and its deep-sea bacterial host. We found that the P2 prophage resides stably in the genome of W3-18-1, but it remains excisable with a low frequency. More importantly, free P2 phage particles are spontaneously produced and released during normal host growth. The presence of the P2 prophage in the W3-18-1 genome enables the host cells to thrive in the deep sea, and the lysis caused by the production this P2 prophage in a small population was observed to increase biofilm formation. In addition, unlike lytic phages, which are generally natural predators that exploit host cells for growth, the production of P2 increases along with the growth of its host. Thus, these studies demonstrated a type of symbiosis between a P2-family prophage and its *Shewanella* host.

### Results

P2-and P4-like prophages are integrated at ssrA in Shewanella. The presence of prophage near the ssrA locus was analyzed in the 98 sequenced Shewanella strains deposited in the NCBI Genome database (Supplementary Table S1 and S2). Among them, we found that 32 strains harbor a prophage at the 3'-end of the ssrA gene (Supplementary Table S2). For the 32 prophages, 24 of them can be classified into two distinct groups (Groups I and II) based on the phylogenetic analysis of the integrases encoded by these prophages (Supplementary Fig. S1 and Supplementary file S2). Shewanella strains that harbor Group I prophages at the ssrA locus include S. putrefaciens W3-18-1, S. baltica OS117, S. baltica 155, S. halifaxensis HAW-EB4, S. xiamenensis BC01, S. sp. GutCb, and S. marina JCM15074 (Fig. 1). Group I prophages contain a conserved phage P2 structural region, L-M-N-O-P-Q (from Sputw3181\_2897 to Sputw3181\_2901), which is a diagnostic signature region of P2-family phages (Casjens and Grose, 2016), and a conserved phage regulatory region containing CI, CII and Cox. In Group II, the integrases share medium to high sequence identity (57-99% with query coverage >96%) with the integrase of the P4-like prophage CP4So of S. oneidensis MR-1 (Fig. 1). The integrases of the Group I prophages share low sequence identity (23-26% with query coverage <50%) with the integrase of CP4So. In addition, the Group II prophages do not possess any characteristic phage structural genes but contain regulatory genes of P4-like phages, such as the *intA* gene encoding integrase and the *alpA* gene encoding excisionase, suggesting that the Group II prophages are mostly cryptic prophages or satellite prophages. Interestingly, one of the prophage attachment sites overlaps the tRNA-like structure at the tail of the *ssrA* gene (**Supplementary Fig. S1**).

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Further genome analysis of P2Sp prophage with sequenced P2-family phages revealed that P2Sp is closer to the Hp1 subfamily of phages, including K139 from *Vibrio cholera* O139 and ΦO18P from *Aeromonas media* O18, than to the P2 subfamily of the enterobacteria P2 phages (**Supplementary Fig. S2A**). In addition to the conserved structural region (L-M-N-O-P-Q) and the regulatory region (CI, CII and Cox), Sputw3181\_2905 (the phage replication protein) and Sputw3181\_2916 (putative integrase) are also conserved in P2 family phages. However, the Sputw3181\_2882-Sputw3181\_2885 and Sputw3181\_2890-Sputw3181\_2894 regions are conserved only in the Hp1 subfamily of phages. Furthermore, P2Sp also carries specific cargo genes, such as those encoding a ParE/YefM toxin-antitoxin system (Sputw3181\_2902/Sputw3181\_2903) and P-loop domain-containing protein (Sputw3181\_2917). Similar results were obtained for the genome comparison of P2Sp prophages with other predicted P2-family prophages in various bacterial hosts (**Supplementary Fig. S2B**).

Identification of mobile genetic elements in W3-18-1. The genome of W3-18-1 contains seven mobile genetic elements (> 10 kb), which comprise 6.20% of its genome. The size and the position of each mobile genetic element are shown in Fig. 2A. P2Sp is inserted at the 3'-end of the *ssrA* gene in W3-18-1, similar to strains O115 and O117 isolated from the Baltic Sea. A lambda family phage (named LambdaSp, 56.3 kb) is inserted in the *sbcB* gene encoding the putative exonuclease I. A P4-like prophage (P4Sp) is inserted in the tRNA<sup>ser</sup> locus. In addition, an integrative and conjugative element, ICE*Spu*PO1 (renamed ICESp), is site-specifically integrated into the 5'-end of the *prfC* locus in W3-18-1 (Pembroke and Piterina, 2006). ICESp belongs to the SXT/R391 family, which is one of the most abundant conjugative elements in bacteria (Marrero and Waldor, 2007; Wozniak *et al.*, 2009). In addition, two genomic islands were also found in W3-18-1. GI*Spu*POI (renamed GISp, 23.0 kb) is inserted in the *trmE* gene encoding a tRNA modification enzyme. A Tn3-family transposon (Tn3Sp) is inserted at a different tRNA<sup>ser</sup> locus from the one that serves as the integration site for P4Sp. Among these mobile genetic elements, only P2Sp and LambdaSp contain phage structural genes that might assemble active phage particles.

To investigate whether W3-18-1 can release phage particles under normal growing conditions, culture supernatant of W3-18-1 was collected at the stationary phase, and the presence of phage genes in the culture supernatant was detected by PCR. As expected, only genes from P2Sp and LambdaSp were detected

(Supplementary Fig. S3). To further quantify the free P2Sp particles produced by W3-18-1, SYBR Gold staining was used to quantify the number of phage in the supernatant of W3-18-1, as previously demonstrated in the quantification of free phage particles in marine environments (Chen *et al.*, 2001). To avoid the effect of lambda-like phage, we deleted the lambda-like prophage (Sputw3181\_2453-2529) in W3-18-1 to construct the W3-18-1  $\Delta$ LambdaSp strain. The number of P2Sp phages released by the  $\Delta$ LambdaSp strain was ~ 10<sup>9</sup> ml<sup>-1</sup> in the stationary phase (Fig. 2B). Next, the culture supernatant of W3-18-1  $\Delta$ LambdaSp was collected to identify P2Sp phages by transmission electron microscopy (TEM) using sucrose-gradient centrifugation. As expected, a P2-family phage with a rigid tail was observed (Fig. 2C), which is different from the lambda-like phage with a flexible tail (Fig. 2D).

**Heterogeneity in P2Sp phage production**. To further visualize the individual host cell that undergoes prophage P2Sp induction in a population, a fluorescently labeled strain W3-18-1 MCP::GFP was constructed in which the gene Sputw3181\_2898, encoding the major coat protein (MCP), was fused in-frame with the *gfp* gene encoding the green fluorescence protein (**Fig. 3A**). Since MCP is needed for the assembly of phage particles, the expression of the fused protein MCP::GFP can be used as an indicator of P2Sp production.

As expected, the majority of cells appeared dim under fluorescence microscopy, suggesting that P2Sp was mostly maintained in a lysogenic state in W3-18-1 (**Fig. 3B**). Noticeably, a subset of cells (< 1%) showed strong <sup>C1</sup> prescent signals, suggesting a faction of cells producing P2Sp phages. Similar results were obtained using another reporter strain, W3-18-1 Cox::GFP, in which the gene Sputw3181\_2910, encoding the P2Sp Cox protein, was fused with *gfp* (**Fig. 3B**). More importantly, the use of time-lapse microscopy to monitor a cell undergoing induction of P2Sp showed the process of cell rupture for cells producing MCP. Cell lysis occurred between 20 min to 40 min with the transition of a rod-shaped cell to a bloated cell and further ruptured at 80 min (**Fig. 3C**). Additionally, the fraction of cells undergoing induction was also quantified using flow cytometry by detecting the

GFP signal in a population of W3-18-1 MCP::GFP and W3-18-1 Cox::GFP cells. The fraction of cells undergoing prophage induction estimated by flow cytometry using each of the two different GFP fusions was ~ 0.1% in a total of  $2 \times 10^5$  cells (**Fig. 3D**). Collectively, these assays demonstrated that P2Sp is spontaneously produced at a frequency of ~ 0.1% in W3-18-1.

**P2Sp phage is produced constitutively and at a rate proportional to the host growth rate.** We also monitored P2Sp phage production along with host growth. An overnight culture of W3-18-1 was reinoculated into fresh LB medium (1:1000), and host growth (CFU ml<sup>-1</sup> and OD<sub>600</sub>) was then measured at different time points. As shown in **Fig. 3E** and **Supplementary Fig. S4**, although P2Sp induction was observed, the growth of W3-18-1 still followed classical growth kinetics and reached stationary phase after 16 h. Noticeably, P2Sp phage production increased with host growth rate (**Fig. 3E** and **Supplementary Fig. S4**). After 24 h of incubation, the concentration of P2Sp phage particles in the culture supernatant was  $4.3 \pm 0.2 \times 10^9$  ml<sup>-1</sup>, and the host cell density was  $7.0 \pm 0.5 \times 10^8$  ml<sup>-1</sup>. The ratio of the phage to the host was calculated at different stages. The average phage-to-host ratio was  $2.9 \pm 0.5$ , with the highest ratio of  $6.1 \pm 0.2$  at 24 h and the lowest ratio of  $0.9 \pm 0.4$  at 6 h (**Fig. 3F**). To determine whether SOS stress can trigger the excision of the prophage, mitomycin C (MMC) was added to the exponentially growing cells. The excision of LambdaSp was upregulated approximately 2000-fold in the presence of  $0.5 \ \mu g \ ml^{-1}$  MMC, reaching up to  $34 \ -38\%$  of cells with LambdaSp excised. In contrast, MMC treatment did not change the frequency of P2Sp excision (**Supplementary Fig. S6**). Unlike CP4So in *S. oneidensis* MR-1, the excision of P2Sp was also not changed at 4, 15 or 25 °C. These results suggest that P2Sp was constitutively produced in a small fraction of host cells during host growth and was noninducible in the SOS response.

**P2Sp prophage excision is promoted by the Cox protein.** Phage integration is a crucial step for the onset of lysogenic conversion, while prophage excision is a critical step for the onset of phage production (Casjens, 2003; Brussow *et al.*, 2004; Fortier and Sekulovic, 2013; Feiner *et al.*, 2015; Menouni *et al.*, 2015). We have

demonstrated that integrase (IntA) and excisionase (AlpA) from the Group II prophages control the integration and excision of the P4-like prophage in S. oneidensis MR-1 (Zeng et al., 2016). For the temperate enterobacteria phage P2, conversion between the lysogenic cycle and lytic cycle is controlled by a multifunctional Cox protein (Yu and Haggard-ljungquist, 1993; Ahlgren-Berg et al., 2009). The P2-family prophage (Group I) in Shewanella contains a regulatory region (Fig. 1); however, the putative regulatory genes share low sequence similarity (29-37%) identity with 39-47% coverage) with the previously reported Cox protein in phage P2. In addition, the Cox proteins of Shewanella isolates (66-76 aa) are shorter than the Cox protein (91 aa) of enterobacteria phage P2 (Fig. **4A**). To test whether the P2-family prophages remain excision-proficient in *Shewanella*, the coding regions of the cox (Sputw3181\_2910), int (Sputw3181\_2916), and a conserved replication gene rep (Sputw3181\_2905) were cloned into pHGE under a Ptac-inducible promoter. PCR was employed to check P2Sp excision using a forward primer P2Sp-F flanking the left attachment site (attL) and a reverse primer P2Sp-R flanking the right attachment site (attR) of the P2Sp prophage (Fig. 4B). Due to the large genome size of P2Sp prophage (34.8 kb) and the limitation of DNA amplification by PCR, PCR products can only be obtained when P2Sp prophage is excised from the host genome. Overexpressing cox or rep via pHGE-cox for 4 h with the addition of 1 mM IPTG led to P2Sp excision, detected by the presence of a clear PCR product with the expected size when the whole prophage was removed (Fig. 4C). No excision band was detected when the integrase gene was overexpressed.

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Furthermore, qPCR was performed to determine the fraction of W3-18-1 cells that underwent P2Sp excision in a total of  $\sim 10^9$  cells. The frequency of P2Sp excision in W3-18-1 cells was low (~ 3 out of  $10^5$  cells) under normal growth conditions, suggesting that P2Sp mostly resides stably in the host genome. As expected, overexpression of the *integrase* did not affect P2Sp excision in the wild-type W3-18-1 strain (**Fig. 4D**). In contrast, the frequency of P2Sp excision increased approximately  $10^3$ -fold when *cox* was overexpressed in the wild-type W3-18-1 strain, reaching up to ~18%. A similar frequency of P2Sp excision was observed when the replication

protein Rep was overexpressed, reaching up to  $\sim 9\%$ . These results suggest that excision of P2Sp is promoted by Cox and Rep. As a negative control, the excision of P2Sp was undetectable after *int* was deleted, indicating that Int is required for the excision of P2Sp. Because rep is functional in P2Sp excision and its analogues are conserved in P2-family prophages (Fig. 1 and Supplementary Fig. S2), a similar qPCR approach that quantifies the numbers of phage circles was performed to test whether P2Sp can replicate after its excision. In theory, the excision of a prophage yields one *attB* on the chromosome and one extrachromosomal copy of the circularized prophage (attP), so without replication, the ratio of attP/attB should be equal to 1 (Fig. 4B). In fact, this ratio reached 106  $\pm$  2 in wild-type W3-18-1 and 101  $\pm$  4 in wild-type W3-18-1 containing an empty plasmid, suggesting that P2Sp is capable of replicating in a subset of wild-type populations (Fig. 4E). In contrast, this ratio decreased to lower than 6 when Int was overexpressed, indicating that Int promoted the reintegration of the P2Sp circles. Furthermore, the ratio of attP/attB decreased to lower than 1 when Cox was overexpressed because overexpression of Cox led to a 30-fold increase in P2Sp replication (attP/gyrB, data not shown) but a 10<sup>3</sup>-fold increase of P2Sp excision (attB/gyrB). In contrast, the ratio of attP/attB was  $111 \pm 5$  when Rep was overexpressed, even though Rep caused a similar level of P2Sp excision compared to Cox (9% vs 18%) (Fig. 4DE). These results suggested that overexpression of Rep promoted the excision and replication of P2Sp in a high proportion of W3-18-1 wild-type cells. As a negative control, the replication form of P2Sp was undetectable in the  $\Delta rep$ background. Together, these observations suggested that the P2Sp prophage retains the ability to excise from the host genome and the ability to replicate. Cox seems to act extensively on excision of P2Sp while Rep not only promotes the excision but also increases the replication of P2Sp.

**P2Sp is required to maintain SsrA function and for host survival in marine environments.** Previous studies showed that genome excision of prophages inserted in critical genes such as tmRNA may alter the function of these genes (Wang *et al.*, 2009; Zeng *et al.*, 2016). To explore the effect of P2Sp excision on *ssrA*, an isogenic

strain, W3-18-1  $\Delta P2Sp^{Ex}$ , was constructed in which the whole prophage was removed in a way that is equivalent to natural P2Sp excision (**Fig. 5A**). Briefly, the  $\Delta P2Sp^{Ex}$  strain was selected using PCR screening from a population of cells overexpressing the Cox protein with plasmid pHGE-*cox*, which can increase the P2Sp excision rate to ~18%. Plasmid pHGE-*cox* was then removed by growing these cells without antibiotics for two passages. The complete removal of integrated P2Sp and the extrachromosomal P2Sp phage circle were confirmed by PCR using primers specifically amplifying the P2Sp genes (**Supplementary Fig. S7**). DNA sequencing analysis of the reconstituted bacterial attachment site (*attB*) in the  $\Delta P2Sp^{Ex}$  strain revealed that P2Sp excision led to a single deletion of a U at the 3'-end of SsrA due to a site-specific recombination event (**Fig. 5AB**). The deletion of this U disrupted a conserved G·U wobble base pair in the tRNA-like domain, which is a critical base pair in determining the function of SsrA (Hou and Schimmel, 1988).

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To determine whether the single deletion of U affects SsrA function *in vivo*, we first compared the physiological changes caused by this excision in  $\Delta P2Sp^{Ex}$  and in the *ssrA* deletion mutant strain. To keep the right attachment site (*attL*) intact, a 158 bp fragment containing the region encoding the SsrA tag sequence was removed to construct an *ssrA* deletion mutant strain,  $\Delta ssrA$  (**Fig. 5A**). The  $\Delta ssrA$  strain displayed increased susceptibility to the miscoding antibiotic gentamicin compared to the wild-type strain when tested at a sublethal concentration (4 µg ml<sup>-1</sup>) at 25 °C (**Fig. 5C**). However, at higher concentrations (e.g., 10 µg ml<sup>-1</sup>), gentamicin inhibited the growth of both the wild-type cells and  $\Delta ssrA$  cells to the same extent (results not shown). These results were expected because it has been reported that SsrA in *E. coli* and *S. oneidensis* MR-1 contributes to the survival of cells exposed to miscoding antibiotics but only at sublethal concentrations (Abo *et al.*, 2002; Zeng *et al.*, 2016). Similar results were obtained when the miscoding antibiotic kanamycin was tested, with reduced  $\Delta ssrA$  survival observed at sublethal concentrations (**Fig. 5C**). In the presence of 10 µg ml<sup>-1</sup> kanamycin, the  $\Delta P2Sp^{Ex}$  strain showed increased susceptibility compared to the wild-type strain, but the increase was comparable to that

observed in the  $\Delta ssrA$  strain, suggesting that P2Sp excision disrupted the quality control function of SsrA in protein synthesis. We recently showed that the disruption of the G·U wobble base pair at the 3'-end of SsrA due to CP4So excision completely abolished the function of SsrA in the *S. oneidensis* MR-1 strain (Zeng *et al.*, 2016). Thus, both P2 and P4 prophages inserted in SsrA abolish the function of SsrA after their excision.

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To explore the effect of P2Sp excision on host physiology, the growth of the  $\Delta P2Sp^{Ex}$  strain was first evaluated. Since most Shewanella strains are believed to have a marine origin, growth was evaluated in LB medium and sea water LB medium (SW-LB, 35‰ salinity), respectively. The growth of  $\Delta P2Sp^{Ex}$  was similar to the wild-type strain in LB medium (Fig. 5D) but was severely inhibited in the SW-LB medium at 25 °C (Fig. 5E). Furthermore, the wild-type SsrA and the variant SsrA<sup>delU</sup> were cloned into the pHGE plasmid and then introduced into the  $\Delta P2Sp^{Ex}$  strain to construct the complement strains, after which bacterial growth was tested (Supplementary Fig. S8). These data showed that expression of wild-type SsrA restored the growth of the  $\Delta P2Sp^{Ex}$  strain, whereas expression of the variant SsrA<sup>delU</sup> failed to restore the wild-type phenotype. As an attached biofilm is critical for prolonged survival at cold temperatures (Tribelli and Lopez, 2011; Zhang et al., 2015; Zeng et al., 2016), we tested the attached biofilm formation of W3-18-1 at 15 °C. Noticeably, the  $\Delta P2Sp^{Ex}$ strain formed much less biofilm than the wild-type W3-18-1 cells at 24 h or 48 h (Fig. 5F). Additionally, similar to the  $\Delta P2Sp^{Ex}$  strain, the  $\Delta ssrA$  strain also showed severe growth defects in SW-LB and reduced biofilm formation (Fig. 5EF). When the wild-type SsrA and the variant SsrA<sup>delU</sup> were complemented using pHGE-based plasmids in the  $\Delta P2Sp^{Ex}$  and in  $\Delta ssrA$  strains, the wild-type SsrA but not the SsrA<sup>delU</sup> variant increased biofilm formation in these two strains (Fig. 5G). These results suggest that the reduced fitness for growth and biofilm formation in the  $\Delta P2Sp^{Ex}$  strain are partially due to the disruption of SsrA, which results from P2Sp excision. These data suggest that SsrA is important for marine bacteria to grow in sea water and excision of P2Sp is harmful to individual bacterial cells living in deep-sea environments by disrupting the function of *ssrA*.

**P2Sp genes promote biofilm formation and secondary transporters.** Since the majority of P2Sp prophage maintains a lysogenic state in the W3-18-1 host, we wondered whether the P2Sp genes directly contribute to host fitness. Thus, we constructed a deletion mutant strain  $\Delta P2Sp^{KO}$  with all 41 P2Sp genes knocked out from W3-18-1 using the *att*-based fusion PCR method (Jin *et al.*, 2013). In contrast to the  $\Delta P2Sp^{Ex}$  strain, the *ssrA* gene was kept intact and functional in the  $\Delta P2Sp^{KO}$  strain (**Fig. 6A**). As shown in **Fig. 6B**, the  $\Delta P2Sp^{KO}$  strain showed a reduction in attached biofilms after 48 h at 15 °C, indicating that the presence of P2Sp genes can enhance biofilm formation. Given that the overexpression of Rep can promote the replication of the P2Sp genome in a high proportion of cells (**Fig. 4DE**), attached biofilms were tested in wild-type W3-18-1 when Rep was overexpressed. As shown in **Fig. 6C**, attached biofilms were significantly increased only after Rep was overexpressed. These results suggest that products of the P2 genes increase biofilm formation.

Furthermore, a whole-transcriptome study was performed to characterize the effect of the P2Sp genes removal from W3-18-1 and to explore the effects of P2Sp on host physiology at the transcriptional level (NCBI SRA database under number PRJNA504380). In stationary growing W3-18-1 wild-type cells, 40 out of the 41 P2Sp genes had signals higher than 50, and one gene, Sputw3181\_2890, which encodes a TraR/DskA family transcriptional regulator, had the lowest signal of 29. Among them, genes encoding the replication protein, phage terminase, phage integrase and the phage tail tape measure protein were highly expressed (signal >1000) (Supplementary Table S4), indicating that P2Sp is actively produced in W3-18-1. For the three P2 regulatory proteins, the expression of the repressor CI was much higher than the expression of CII or Cox, explaining the lysogeny of P2Sp in the majority of host cells. Additionally, P2Sp-specific genes with unknown functions (e.g., Sputw3181\_2881, 2913, 2916 and 2917), which are not conserved in other P2-family prophages, were also highly expressed (signal >1000). Hence, the prophage genes may actively influence biofilm formation and other physiological functions of the host cells.

Upon deletion of the P2Sp prophage, the majority of the host genes (> 99.9%) were not differentially expressed. Only the gene encoding the secondary transporter system and the gene Sputw3181\_2825 with a hypothetical function were significantly repressed by removal of the P2Sp genes (Supplementary Table S4). Furthermore, these results were corroborated by a qRT-PCR analysis showing that the complete loss of the P2Sp genes repressed the expression of the *dctPQM* operon and a hypothetical gene Sputw3181\_2825 (Fig. 6D). The *dctPQM* operons encode secondary transporters and the tripartite ATP-independent periplasmic (TRAP) transporter system, which has been shown to be an important transporter in marine bacteria living in extreme environments (Kelly and Thomas, 2001; Mulligan *et al.*, 2011). Overall, these analyses showed that the loss of P2Sp affected genes involved in substrate transport and biofilm formation.

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**P2** phages are prevalent in Proteobacteria and active in the natural microbial community. To investigate the distribution of P2 prophages, we searched against 5176 fully sequenced genomes in the KEGG Sequence Similarity Database (SSDB) (Kanehisa *et al.*, 2017) to collect sequences similar to that of the MCP of P2Sp (**Supplementary file S3**). A total of 556 bacterial and 36 viral sequences with hits to pfam05125 (Phage\_cap\_P2) were retained for further analysis (**Supplementary files S4** and **S5**). All SSDB searches yielded P2-family MCPs that were identified in proteobacterial genomes and formed two phylogenetically distinct groups, Hp1 and P2 (**Fig. 7** and **Supplementary Fig. S9A**), which were both found in different species belonging to the same genus, including *Shewanella*. Analysis of the locations of prophages and *ssrA* genes showed that 171 prophages, including 58 P2-family prophages, were integrated into *ssrA* (**Fig. 7**), which may suggest that hijacking *ssrA* is important for the mutualism of P2-family prophages. The integration sites of P2 into *Shewanella* include *ssrA*, *dusA* (encoding tRNA-dihydrouridine synthase A), *rluA* (encoding tRNA pseudouridine 32 synthase) and other loci. For example, *S.* sp. MR-7 harbors a P2-family prophage at the *dusA* locus and *S. baltica* OS678 harbors a P2-family prophage at the *rluA* locus. Moreover, prophage genome sizes and the number of prophages in bacterial

strains showed no significant differences between these 58 P2-family prophages and other P2-family prophages (**Supplementary Fig. S9BC**). Further searching in the Integrated Microbial Genomes and Microbiomes (IMG/M) database revealed the existence of P2-family MCPs in the phylum Firmicutes, and these prophages also possess the conserved region L-M-N-O-P-Q (**Supplementary Fig. S10**), although P2-family MCPs were previously not found outside the phylum Proteobacteria by BLASTP searching (Casjens and Grose, 2016).

Moreover, public metatranscriptomes and viral metagenomes were used to investigate P2-family prophages in natural microbial communities. Searching the aquatic metatranscriptomes in the IMG/M database yielded 74 P2-family MCP fragments (**Supplementary File S6**). Some deep-sea fragments derived from the Global Malaspina Expedition project (Duarte, 2015), including viral communities from the surface down to 4000 m depth of the Atlantic, Indian, and Pacific tropical and subtropical oceans, showed high similarities with P2 MCP proteins in *Shewanella, Halomonas, Erythrobacter* and *Psychrobacter* spp. by BLASTP searching. Further BLASTN searching against the viral metagenomes of the Global Malaspina Expedition project identified nearly identical DNA sequences in the metatranscriptomes (**Supplementary File S6** and **Supplementary Fig. S11**). These results indicated that the P2-family prophages in *Shewanella, Halomonas, Erythrobacter* and *Psychrobacter* spp. might be active and spontaneously induced to form viral particles in the natural microbial communities in the deep sea.

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### Discussion

This study presents the following lines of evidence to show that P2 has established a symbiotic relationship with its host W3-18-1: (i) P2Sp mostly resides stably in the host genome and is noninducible upon the SOS response; (ii) P2Sp phage particles are spontaneously produced and released in a small fraction of host cells under normal growth conditions; (iii) P2Sp excision abolishes the function of SsrA by disrupting a wobble base pairing; (iv) the presence of P2Sp in the host genome enables host cells to grow in a marine environment by maintaining active SsrA; and (v) upon excision, P2Sp promotes biofilm formation and the expression of a secondary

transporter system. Taken together, these results clearly show that the establishment of lysogeny benefits both the P2 phage and its bacterial host (**Fig. 8**).

The integration of temperate phages provides the bacterial host with multiple beneficial traits over its noninfected counterparts, which has been previously demonstrated in *E. coli* and other pathogenic bacteria (Refardt, 2011; Roossinck, 2011; Bobay *et al.*, 2014; Knowles *et al.*, 2016; Argov *et al.*, 2017). For example, lambda, Mu, P1, and P2 prophages increase the growth of *E. coli* under glucose-limited growing conditions (Edlin *et al.*, 1975; Edlin *et al.*, 1977), and cryptic prophages provide multiple benefits for *E. coli* under stressed conditions such as oxidative and osmotic stress (Wang *et al.*, 2010). Additionally, the temperate phage SopEΦ promotes the production of inducible nitric oxide synthase in *Salmonella* (Lopez *et al.*, 2012), thus increasing fitness during anaerobic respiration inside host cells (Barrett and Riggs, 1982; Lopez *et al.*, 2012). The prophage Gifsy-2 can also give the host *Salmonella* a competitive advantage by killing competitors and by providing immunity (Bossi *et al.*, 2003). Recent studies of the deep-sea filamentous prophage SW1 in *S. piezotolerans* WP3 revealed that prophage can adjust the fitness of the host cells in the deep-sea environment by regulating swarming (Jian *et al.*, 2013). Hence, our results support the previous hypothesis that forming a lysogen is beneficial for both phages and bacteria in a deep-sea environment that does not have enough nutrients to support rapid host growth and/or phage propagation (Jiang and Paul, 1998; Weinbauer *et al.*, 2003; Mizuno *et al.*, 2016).

It can be concluded from this study that a bacterial host can adopt a behavioral strategy that can enhance fitness in social groups, despite being costly to the individual in terms of growth, with the help from its competitor, the phage. From the perspective of the individual bacterium, the presence of P2Sp in the genome of W3-18-1 is critical for its survival through its use of a functional SsrA that is required in the marine environment (**Fig. 8**). SsrA contributes to the recycling of ribosomes stalled on aberrant mRNAs and adds tags to misfolded or truncated peptides for degradation, ensuring proper protein synthesis in the cells by recycling ribosomes (Ueda *et al.*, 2002).

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Inactivation of SsrA is usually nonlethal in LB or during other robust growing conditions but leads to increased susceptibility to stress, which generates more aberrant mRNAs (Muto *et al.*, 2000). The results from this study demonstrate that not only the deletion of *ssrA* but also the disruption of the wobble base pairing of SsrA greatly reduce the ability of W3-18-1 to grow in SW-LB (high salinity) rather than LB medium (low salinity). Thus, it is likely that conditions with higher salinity are prone to produce more aberrant mRNAs than low salinity conditions for W3-18-1. Further investigation is needed to elucidate the role of SsrA in marine bacteria. These results further cement the importance of maintaining a lysogenic state once P2 is integrated into the genome of marine bacteria. Thus, the spontaneous production and release of P2Sp phages in a small fraction of host cells is detrimental for the individual lysed cells; however, such action could also be beneficial at the population level. For the phage, the production and release of progeny in a subpopulation of host cells increases the reproductive success of P2 by providing opportunities to find new hosts in the surrounding environment. Nevertheless, the majority of P2-family prophage resides in the host chromosome, increasing its reproductive success in conditions in which potential hosts suffer from scarcity in the surrounding environment, including the deep-sea zone.

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Previous bioinformatics analysis revealed that P4-family integrases were found near the *ssrA* locus in *E. coli*, while P2-family integrases were found near the *ssrA* locus in *Salmonella* (Williams, 2003). Enterobacteria phage P2 and its satellite P4 phage are both classified in the family Myoviridae and the order Caudovirales, featuring a contractile sheath. However, P4 depends upon late genes of P2 for its propagation (Six and Klug, 1973; Lindqvist *et al.*, 1993; Briani *et al.*, 2001). In this study, our analysis demonstrated that integrases near the *ssrA* locus in *Shewanella* can be divided into at least two families, P4 and P2. P4-like prophages vary greatly in genome size and cargo genes, while P2-family prophages are relatively uniform in genome size and all carry conserved phage structural genes (**Fig. 1**). By studying P4-family prophages in *S. oneidensis* MR-1 and P2-family prophages in W3-18-1, different phage-host relationships were revealed in *Shewanella* strains (**Fig. 8**). First, we demonstrated

that prophage induction differs for P4-and P2-family prophages. For MR-1, which was isolated from the surface sediment (63 m beneath the lake surface) of Lake Oneida, cold temperature specifically induced P4 prophage excision (Zeng et al., 2016). In contrast, temperature did not affect P2 prophage excision in W3-18-1. In addition, the excision or induction rate of P2Sp was not changed under various conditions, including during the SOS response. Moreover, we previously reported that the histone-like nucleoid structuring protein (H-NS) could regulate P4-like prophage excision via binding to the promoter of the excisionase gene in MR-1. Although the H-NS protein in MR-1 and W3-18-1 shares high sequence identity (92% with 99% coverage), the H-NS protein in W3-18-1 does not control P2 prophage excision (Supplementary Fig. S12). Second, the consequences of prophage excision in host bacteria differ between P2 and P4. P2Sp prophage excision greatly reduces the survival of W3-18-1 in the marine environment, while P4 prophage excision only slightly reduces MR-1 growth, showing the importance of maintaining P2 prophages integrated into the W3-18-1 genome. Third, even though both P2-and P4-family prophages can excise from the host chromosome, the fate of the phage after excision is also different. P4, which is a domestic prophage, only forms an extrachromosomal circle but cannot replicate on its own. Once excised, the extrachromosomal circle of P4-like prophage requires a toxin-antitoxin system for stability (Yao et al., 2018). Since the P4-like prophage in S. oneidensis MR-1 is cryptic and incapable of forming phage particles, this domesticated prophage has become a regulatory switch for host behavior by altering host genes under specific conditions. Thus, the relationship between the P4-like cryptic prophages and their hosts is parasitic instead of mutualistic. In contrast, we show here that P2-family prophage can replicate and produce phage particles after excision. Host bacteria benefits from P2 prophages at both the population level (increased biofilm formation) and at the individual level (functional SsrA). The released phage particles of P2Sp gain new opportunities for finding other hosts in the surrounding environments. Thus, we show a P2-family prophage and its host established a mutualistic bacteria-phage relationship. Analysis of the locations of prophages and the 3'-end of ssrA genes

suggests that hijacking the host tmRNA is important for the mutualism of P2-family prophages.

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An increasing number of studies have provided evidence that prophages directly affect different stages of biofilm formation and increase the overall fitness of bacterial communities, including Pf4 in Pseudomonas aeruginosa PAO1 (Rice et al., 2009), CP4-57 and DLP12 in E. coli K-12 (Wang et al., 2009), SV1 in Streptococcus pneumoniae (Carrolo et al., 2010) and lambda and Mu in S. oneidensis MR-1 (Godeke et al., 2011). Biofilm development involves complex group behaviors in which phages and bacterial hosts cooperate or compete. During biofilm formation, a fraction of cells lyse and releases eDNA, which is an important component of biofilms (Harmsen et al., 2010). Phage- or phage-like structures spontaneously induce cell lysis are frequently reported to be involved in the bacterial biofilm formation process (Godeke et al., 2011; Turnbull et al., 2016). Although the underlying molecular mechanism of spontaneous prophage induction remains unknown, the lysis of individual cells may benefit the population through the release of "public goods" such as eDNA (Vasse et al., 2015; Attar, 2016; Saucedo-Mora et al., 2017). Moreover, P2Sp-encoded genes also increase biofilm formation, and many of them are expressed at intermediate levels in wild-type W3-18-1. However, the functions of these genes remain unexplored (e.g., Sputw3181 2917 encodes a KAP P-loop domain-containing protein, and Sputw3181 2902/2903 encodes a putative toxin-antitoxin pair). Thus, it is likely that these genes participate in biofilm formation, but further studies are needed to investigate the functions of these P2Sp genes. Therefore, P2 may participate in deep-sea biogeochemical cycles, and more studies are required to unveil additional ecological roles of temperate phages in deep-sea ecosystems.

### **Experimental Procedures**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in **Supplementary Tables S1 and S2**. Luria-Bertani (LB) was used for growing *Escherichia coli* strains, and sea-water LB (SW-LB) was used for growing *Shewanella* strains unless otherwise indicated. *Shewanella* strains were cultured at 25 °C

and *E. coli* strains were cultivated at 37 °C unless otherwise indicated. To facilitate genetic manipulation, the W3-18-1  $\Delta pstI\Delta pstM$  strain, in which the two genes encoding the restriction-modification system were deleted, was used throughout this study (Qiu *et al.*, 2013). Kanamycin (50 µg ml<sup>-1</sup>) was used to maintain the pHGE-based plasmids. Ampicillin (50 µg ml<sup>-1</sup>) and gentamycin (15 µg ml<sup>-1</sup>) were used to maintain the pHGM01-based plasmids. Ampicillin (50 µg ml<sup>-1</sup>) was used to maintain the pMD19-T-based plasmids. For the auxotrophic *E. coli* strain WM3064, 2,6-diamino-pimelic acid (DAP) was added at a final concentration of 0.3 mM. The primers used to construct plasmids are listed in **Supplementary Table S3**.

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**Phage purification.** Phage particles were purified using the polyethylene glycol (PEG)-mediated precipitation method as previously described (Bertani and Bertani, 1970; Sambrook and Russell, 2006). Culture supernatant of W3-18-1 was collected by centrifugation (17 000 × g, for 15 min) after growing cells for 48 h. The supernatant was supplemented with NaCl to a final concentration of 1 M and then incubated at 4 °C for 1 h. Subsequently, the supernatant was filtered through a 0.22-µm filter (Merck Millipore, Darmstadt, Germany) to remove bacterial cells but pass phages, given that the myovirus P2 has an assembled capsid of 60 nm in diameter and a 135 nm contractile tail (Dokland *et al.*, 1992; Christie and Calendar, 2016), and then treated with DNase I (1 µg ml<sup>-1</sup>) at 37 °C. The filtrate was supplemented with PEG 8000 at a final concentration of 10% (m/v) and incubated overnight at 4 °C. Phage particles were pelleted by centrifugation at 17 000 × g for 45 min at 4 °C. Then, the , ge pellets were resuspended in 1 ml of SM buffer (100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl) and subsequently subjected to density gradient centrifugation (250 000 × g, for 2.5 h at 4 °C) to remove PEG as previously described (Bertani and Bertani, 1970). Finally, purified phage particles were negatively stained with 2% phosphotungstic acid (pH 6.8) and subsequently observed using an FEI G2 F20 TWIN electron microscope at 120 kV.

Construction of GFP fused reporter strains. To visualize individual cells that undergo P2Sp induction in a

population of cells, two reporter strains (W3-18-1 MCP::GFP and W3-18-1 Cox::GFP) were generated by the in-frame insertion of the gfp gene into the 3'-terminus of the major capsid protein gene mcp, Sputw3181\_2898 or the multifunctional gene cox (encoding Cox that functions as an excisionase and regulator for late gene transcription). Primers used for the construction of the reporter strains are listed in Supplementary Table S3. Genomic DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) and used as a PCR template. To create W3-18-1 MCP::GFP, the 1366 bp upstream and 1316 bp downstream fragments were amplified and joined through fusion PCR. In-frame insertion of gfp in W3-18-1 was performed by the att-based fusion PCR method (Jin et al., 2013). The fused fragments were introduced into the suicide plasmid pHGM01 using BP Clonase (Thermo Fisher Scientific, Waltham, MA, USA) to create pHGM01-MCP. A gfp-containing NotI-SpeI fragment was cloned and inserted into the pHGM01-MCP plasmid to generate pHGM01-MCP::GFP. Next, the GFP-fused constructs were transferred into W3-18-1 via conjugation using E. coli WM3064 as the donor strain. The transconjugants were selected using gentamycin resistance and further verified by PCR. Then, the verified colonies were cultured in LB medium in the absence of NaCl and plated on LB containing 10% sucrose. Finally, the correct colonies of W3-18-1 MCP::GFP, both gentamycin-sensitive and sucrose-resistant, were screened by PCR for insertion of the gfp gene. W3-18-1 Cox::GFP was constructed following a similar procedure except that the 1147 bp upstream and 1 452 bp downstream fragments of the cox gene were amplified and joined through fusion PCR to create pHGM01-Cox::GFP. Finally, all the correct reporter strains were confirmed by PCR and sequencing of the insertion region.

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**Quantification of phage particles using SYBR Gold staining.** To quantify the number of phage particles in the culture supernatant of W3-18-1, the phage particles were stained with SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Noble and Fuhrman, 1998; Chen *et al.*, 2001). Culture supernatant was collected by centrifugation (8,000 rpm for 2 min). The supernatant was digested with DNase I at a final

concentration of 1 µg ml<sup>-1</sup> at 37 °C for 1 h and subsequently filtered through 0.22-µm filters (Merck Millipore, Darmstadt, Germany). Then, 60 µl of the filtrate was resuspended in 950 µl of SM buffer and filtered onto a 0.02-µm Whatman Anodisc filter (Thermo Fisher Scientific, Waltham, MA, USA). Finally, phage particles on the filter were stained with SYBR Gold, and the filters were mounted on glass slides and observed with a fluorescence microscope (Carl Zeiss, Jena, Germany).

Construction of deletion mutants and plasmids of W3-18-1. In-frame deletion of genes in W3-18-1 employed the *att*-based fusion PCR method (Jin *et al.*, 2013). The primers used in the construction of deletion mutants are listed in **Supplementary Table S3**. For example, to create  $\Delta P2Sp^{KO}$ , in which all 41 genes of P2Sp (Sputw3181\_2877-Sputw3181\_2917) were knocked out without affecting the neighboring gene *ssrA*, a 628-bp fragment (upstream of gene Sputw3181\_2877) and a 937-bp fragment (downstream of Sputw3181\_2917) were amplified and joined through fusion PCR. The fused fragments were introduced into the plasmid pHGM01 using BP Clonase. Next, the pHGM01-derived plasmids were transformed into *E. coli* WM3064 cells and then transferred into W3-18-1 cells via conjugation. By using a similar screening procedure to that described previously in GFP-fused reporter strain construction, the correct  $\Delta P2Sp^{KO}$  colonies were obtained after PCR verification. By using the same deletion method, the strain  $\Delta LambdaSp$ , in which all 77 genes (Sputw3181\_2453-Sputw3181\_2529) were knocked out, and the strain  $\Delta ssrA$ , with a deletion of the 158-bp ion covering the tag sequence, were constructed. All the mutation colonies were confirmed by PCR and sequencing of the mutated region. To overexpress genes in W3-18-1 cells, the full coding regions of the *cox* gene (Sputw3181\_2910), replication gene (Sputw3181\_2905), and the integrase gene (Sputw3181\_2916) were cloned into plasmid pHGE and used.

**Flow cytometry.** The proportion of cells that underwent P2Sp induction was assessed using the BD Accuri C6 Plus flow cytometer by detecting the GFP signal in a population of W3-18-1 MCP::GFP or W3-18-1 Cox::GFP

cells. Briefly, cells were diluted to  $1 \times 10^{6}$  CFU ml<sup>-1</sup> with filtered 0.85% NaCl. Measurements were performed on a logarithmic scale based on the following parameters: 533/30 nm bandpass filter for GFP; run limits for 1 000 000 events; low flow rate (14 µl min<sup>-1</sup>) and a threshold set (50) on forward scatter to allow the discrimination of cell debris.

**Phage quantification using qPCR.** The amount of P2Sp phage release in W3-18-1 was quantified based on the quantification of the amount of P2Sp-specific *cox* in the culture supernatant, following previously published qPCR assays for this purpose (Imamovic *et al.*, 2010; Rooks *et al.*, 2010; Ankrah *et al.*, 2014). Here, a medium copy number plasmid pET28b was used to generate a standard curve for using the *cox* gene in qPCR assays (**Supplementary Fig. S5**). The coding region of *cox* was cloned into pET28b. The plasmid pET28b-*cox* was purified from overnight culture of *E. coli* BL21, and the concentration of the plasmid was quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). To determine the amount of *cox* gene pET28b-*cox*, the following formula was used: [concentration of pET28b-*cox* (ng  $\mu$ l<sup>-1</sup>/molecular weight (ng mol<sup>-1</sup>)] × 6.022 × 10<sup>23</sup> molecules mol<sup>-1</sup> = number of molecules of pET28b-*cox*  $\mu$ l<sup>-1</sup> (Rooks *et al.*, 2010). Next, serial decimal dilutions of this plasmid DNA were prepared as qPCR templates to generate the standard curve. Accordingly, the standard curves were generated by plotting the Ct value of each dilution against the corresponding log gene copy number (**Supplementary Fig. S5**). To quantify the released P2Sp phage, the culture supernatant of W3-18-1 was ired through 0.22-µm filters (Merck Millipore, Darmstadt, Germany) and digested with DNase I.

Screening of the  $\Delta P2Sp^{Ex}$  strain. The P2Sp-excised strain  $\Delta P2Sp^{Ex}$  was obtained by PCR-based screening using W3-18-1 wild-type cells overexpressing *cox* (primers listed in **Supplementary Table S3**). Briefly, W3-18-1 cells harboring pHGE-*cox* were induced with 1 mM isopropyl- $\beta$ -d-thiogalactoside (IPTG) for 4 h starting at a turbidity of 1.0 at 600 nm. To screen for cells that underwent excision of prophage P2Sp, PCR was performed using the primer pair P2Sp-F/R to detect the presence of a 2.0-kb PCR product, which was obtained only after the removal

of P2Sp prophage from the host chromosome of W3-18-1. To obtain the  $\Delta P2Sp^{Ex}$  strain that lacks the P2Sp phage circle, cells were further cultured in LB for 24 h and screened for the complete loss of the P2Sp genome by detecting the loss of the *cox* gene and the replication gene (Sputw3181\_2905). Finally, pHGE-*cox* plasmid was removed by growing these cells without antibiotics for two passages, and the removal of the plasmid was confirmed by the absence of kanamycin resistance in the  $\Delta P2Sp^{Ex}$  strain.

**Quantification of the frequency of prophage excision.** The frequency of prophage excision and the extrachromosomal phage circle number were quantified using quantitative PCR (qPCR) method as previously described (Wang *et al.*, 2010; Liu *et al.*, 2015) and primers listed in **Supplementary Table S3**. Total genomic DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). The number of total cells was quantified by a chromosomal single-copy reference gene, *gyrB*. The fraction of cells with P2 excised was quantified using the primer pair P2Sp-attB-F/R that specifically amplifies the *attB* site after P2 excision. The faction of extrachromosomal phage circles was quantified using the primer pair P2Sp-attB-F/R that specifically amplifies the *attP* site after P2 forms circle. The qPCR reaction was performed on the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, Rockford, USA). The frequency of prophage excision was calculated as the ratio of *attB/gyrB*, and the phage circle was calculated as the ratio of *attP/attB* using the  $2^{-\DeltaACT}$  method as viously described (Pfaffl, 2001; Zeng *et al.*, 2016).

**RNA extraction, transcriptome sequencing and quantitative real-time reverse-transcription PCR** (**qRT-PCR**). Strains of W3-18-1 and  $\Delta$ P2Sp<sup>KO</sup> were grown overnight in LB medium with shaking at 15 °C until the optical density at 600 nm reached 5.5~6.0. Then, the cells were collected, and the total RNA was extracted using the RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China). Transcriptome sequencing was performed on an Illumina HiSeq 2500 platform, and paired-end reads were generated. Additionally, the raw data were

submitted to the NCBI SRA database under number PRJNA504380. Furthermore, to validate the expression profiles of the unique genes, quantitative real-time reverse-transcription PCR (qRT-PCR) was performed. For the reverse transcriptase reactions, 50 ng RNA was used as templates with HiScript<sup>®</sup> II QRT SuperMix (with gDNA wiper) (Vazyme Biotech, Nanjing, China). Primers for qRT-PCR are listed in **Supplementary Table S3**. The housekeeping gene Sputw3181\_*R0001* (16S rRNA gene) was used to normalize the gene expression data.

**Biofilm assay.** Attached biofilm formation was stained with 0.1% crystal violet and measured in 96-well polystyrene plates (Corning Costar, Cambridge, MA, USA) as previously described (Fletcher, 1977; Pratt and Kolter, 1998). *Shewanella* strains were grown statically in LB medium for the indicated time periods. Then, the cells were measured at 620 nm before being stained with crystal violet. Afterwards, the stained cells were measured at 540 nm. Biofilm formation was normalized by bacteria growth. For each strain, three colonies were used for each assay.

**Phylogenetic analysis of the distribution of P2-family prophages.** P2-family MCP homologs were obtained using the KEGG SSDB search (Kanehisa *et al.*, 2017) in June of 2018 (**Supplementary file S3**). Sequences with bit scores greater than 50 were collected and further annotated with InterProScan v5.30-69.0 using the pfam option (Jones *et al.*, 2014). Finally, 556 bacterial and 36 viral sequences with hits to pfam05125 (Phage\_cap\_P2) were obtained (**Supplementary files S4** and **S5**) and cross-referenced to the NCBI database through the KEGG ArrI service. These P2-family MCPs and their corresponding genomes were downloaded from the NCBI database and reannotated using PROKKA v1.12 (Seemann, 2014). Prophages were predicted using PHASTER (Arndt *et al.*, 2016). The locations of *ssrA* genes, P2-family MCP genes and prophages in genomes are summarized in **Supplementary file S4**. The maximum likelihood tree was inferred with IQ-TREE v1.5.5 with parameters (-st AA -m TEST -bb 1000 -alrt 1000) (Nguyen *et al.*, 2015). This ML tree was rooted to split the Hp1 and P2 groups and further annotated by the iTOL tool (Letunic and Bork, 2016). Genomes of P2-family phages and prophages were

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compared using the CGView Comparison Tool (Grant et al., 2012). To investigate whether P2-family prophages were active in natural ecosystems, protein fragments related to pfam05125 in 1320 aquatic metatranscriptomes in the IMG/M database (October 19, 2018) (Chen et al., 2019) were extracted and annotated by BLASTP against the nonredundant NCBI protein database. Most of the metatranscriptomic sequences were derived from the study of deep ocean microbial communities from the Global Malaspina Expedition. Therefore, the metatranscriptomic sequences were used as queries to perform BLASTN searching against viral metagenomes derived from the same tir. study to explore whether P2-family prophages could form viral particles in the deep sea.

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### **Figure legends**

**Fig. 1. Genetic features of representative** *ssrA***-associated prophages in** *Shewanella* **strains.** P2-family (Group I) and P4-family (Group II) prophages were organized and aligned by blastn using Easyfig (version 2.1). The regions of >60% identity are marked by gray shading. The left and right attachment sites within the host chromosome are indicated by red vertical bars. Arrows represent the relative position and transcriptional direction of the open reading frames (ORFs). Predicted gene functions are indicated by the color key featured at bottom. The genome of P2Sp spans from Sputw3181\_2877 to Sputw3181\_2917. The number on top of the integrase shows the amino acid sequence identity of the indicated integrase with the integrase of CP4So in MR-1.

Fig. 2. P2Sp phage particles can be released by W3-18-1. (A) Size and position of the seven mobile genomic islands in the genome of W3-18-1. (B) Fluorescence microscopy images of SYBR Gold-labeled P2Sp phage particles produced by the  $\Delta$ LambdaSp strain. Phage-containing supernatants were collected at stationary phase (OD<sub>600</sub>~ 6.0). A 60 µl aliquot of supernatant was filtered and stained with SYBR gold. (C) Transmission electron micrograph of P2Sp. (D) Transmission electron micrograph of LambdaSp. Phage particles were negatively stained with phosphotungstic acid in C and D.

**Fig. 3. P2Sp phage is constitutively produced in W3-18-1. (A)** Schematic of the reporter system of P2Sp induction. (**B**) Two reporter strains, W3-18-1 MCP::GFP and W3-18-1 Cox::GFP, were constructed to visualize the host cells undergoing P2Sp induction. Induction of P2Sp is indicated by the intracellular production of MCP-GFP or Cox-GFP fusion protein. (**C**) Overlay images of phase contrast and GFP channel depicting the induction of P2Sp in a small fraction of W3-18-1 MCP::GFP cells. Frames show a cell that was undergoing P2Sp induction and other normal growing cells over 120 min. (**D**) Dot plots of two GFP reporter strains using the BD Accuri C6 flow cytometer. Cultures of the GFP reporter strain, W3-18-1 MCP::GFP, W3-18-1 Cox::GFP, and the W3-18-1 strain (negative control) were taken at an OD<sub>600</sub> of 6.0. GFP-A is plotted against SSC. The gate labeled "Bac" was set to define the population of bacteria cells. The gate labeled "GFP" was set to define the population of cells that were undergoing P2Sp induction by detecting MCP-GFP or Cox-GFP. (**E**) Log-transformed P2Sp gene (*cox*) copy number in the culture supernatants of W3-18-1 and the viability (CFU ml<sup>-1</sup>) of W3-18-1 cells. (**F**) Log-transformed P2Sp gene (*cox*) copy number released in the supernatants *versus* CFUs of W3-18-1 cells. The results in **D-F** are representative images of three independent experiments.

**Fig. 4. P2-encoded Cox promotes the excision of prophage P2Sp.** (**A**) Amino acid sequence alignment of putative Cox proteins from *E. coli* O157 EDL933, *E. coli* O157 Sakai, seven *Shewanella* strains including *S. putrefaciens* W3-18-1, *S. baltica* OS117, *S. baltica* OS155, *S. halifaxensis* HAW-EB4, *S.* sp. GutCb, *S.* sp. MR-7 and *S. xiamenensis* BC01, or two *Vibrio* strains including *V.* sp. AND4 and *V. alginolyticus* ANC4-19 with the Cox from enterobacteria phage P2. (**B**) Schematic representation of the excision of prophage P2Sp from the W3-18-1 genome. (**C**) Ethidium bromide-stained 1% agarose gel of PCR products to detect the excision of P2Sp. PCR products flanking *attB* (2 009 bp) were amplified using primers P2Sp-F and P2Sp-R. PCR products of the *gyrB* bands were amplified using primers gyrB-F and gyrB-R. Lanes: M, DNA marker; E, W3-18-1 cell containing an empty plasmid pHGE; *int*, W3-18-1

containing pHGE-*int* (Sputw3181\_2916) plasmid; *cox*, W3-18-1 containing pHGE-*cox* (Sputw3181\_2910) plasmid; *rep* W3-18-1 containing pHGE-*rep* (Sputw3181\_2905) plasmid. (**D**) Excision frequency of P2Sp in W3-18-1 or its derived strains. Genes of *integrase*, *cox* or *replication* were overexpressed for 24 h using pHGE plasmid with the addition of 1 mM IPTG. (**E**) Phage P2Sp circle number in W3-18-1 or its derived strains. Genes of *integrase*, *cox* or *replication* were overexpressed for 24 h using pHGE plasmid with the addition of 1 mM IPTG. (**E**) Phage P2Sp circle number in W3-18-1 or its derived strains. Genes of *integrase*, *cox* or *replication* were overexpressed for 24 h using pHGE plasmid with addition of 1 mM IPTG. Experiments were performed with three independent cultures, and error bars indicate standard error of mean in **D** and **E**. Significant changes are marked with three asterisks for p < 0.0001 and four asterisks for p < 0.0001.

**Fig. 5.** The function of *ssrA* is disrupted after P2Sp excision. (A) Schematic representation of excision of P2Sp from W3-18-1 and two W3-18-1-derived mutants. ΔP2Sp<sup>Ex</sup> is equivalent to a natural P2Sp-excised strain that was generated by overexpressing *cox*. Δ*ssrA* was generated by in-frame deletion of a 158-bp region containing the tag sequence of the *ssrA* gene shown in (B). (B) Change in the tail of *ssrA* before and after excision of P2Sp. The sequences of *attL/attR* and the tail of *ssrA* are shown in Fig. S1. P2Sp excision causes a deletion of U at the 3'-end of *ssrA*. (C) Susceptibility assay of the intact *ssrA* stains (W3-18-1) and *ssrA* mutants (ΔP2Sp<sup>Ex</sup> and Δ*ssrA*) to sublethal concentrations of gentamycin (4.0 µg ml<sup>-1</sup>) and kanamycin (10 µg ml<sup>-1</sup>). Cultures in the exponential growth phase were adjusted to an OD<sub>600</sub> of 1.0 and then diluted to generate 10-fold serial dilutions. Finally, 10 µl of each dilution was plated onto LB (NC, negative control) or antibiotic-containing LB plates. Growth of the W3-18-1, ΔP2Sp<sup>Ex</sup> and Δ*ssrA* at 15 °C. (G) Attached biofilm formation of ΔP2Sp<sup>Ex</sup> and Δ*ssrA* overexpressing the wild-type *ssrA* or the variant *ssrA<sup>delU</sup>* at 15 °C. Experiments were performed with three independent cultures, and error bars indicate standard error of mean in **F** and **G**. Significant changes are marked with one asterisk for *p*< 0.05 and two asterisks for *p* < 0.01.

Fig. 6. P2Sp genes promote biofilm formation and secondary transporter expression. (A) Schematic representation of the prophage P2Sp gene deletion strain  $\Delta P2Sp^{KO}$  stain. All 41 genes of P2Sp were knocked out without affecting the neighboring gene *ssrA*. (B) Attached biofilm formation of wild-type W3-18-1 and  $\Delta P2Sp^{KO}$  at 15 °C. (C) Attached biofilm formation of W3-18-1 at 15 °C when *int, cox,* or *rep* was overexpressed. (D) Transcripts of the secondary transporter system (*dctPQM*) and Sputw3181\_2825 (labeled as Sp\_2825) in the W3-18-1 and  $\Delta P2Sp^{KO}$  strains at 15 °C. Expression of the

16S ribosomal RNA gene Sputw3181\_*R0001* was used to normalize the total RNA in different samples. Data are from three independent cultures, and one standard deviation is shown in B and C. Significant changes are marked with one asterisk for p < 0.05.

**Fig. 7. Phylogenetic tree with the maximum likelihood method of P2-family MCP homologs.** The tree was rooted to split the Hp1 and P2 groups. Tree leaves of P2-family MCPs in phages are labeled. The color strips show the taxonomic group. The red filled circles and empty circles show the relationships of P2-family prophages and *ssrA* genes. The blue filled circles and empty circles show three or two P2-family MCPs in one bacterial strain, respectively, and green filled circles show two P2-family MCPs in one prophage. Two groups of P2-family MCPs in *Shewanella* spp. are highlighted in red.

**Fig. 8. Interplay between prophage and its host.** Both P2 (P2Sp from W3-18-1) and P4 (CP4So from MR-1) prophage insert in the *ssrA* loci in the genome of their hosts. P2 can be spontaneously induced and released, which benefits both the phage and the bacterial host at the cost of a small bacterial population. The induction of P4 was manipulated under the control of the host cells. P4 induction will benefit only the bacterial population at the cost of phage depletion.



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Α α3 α4 η1 0000000000000 000000000 60 70 80 a1 00000000 P2 EDL933 Sakai W3-18-1 OS117 GUICb MR-7 BC01 O395 AND4 ANC4-19 AYESRPKEIRDGWLMWLGLGEPR PQSASGRAGE QIAT QIAT RAKM ALANS AEI D В attB/gyrB P2Sp (34 784 bp) 10 \*\*\*\* W3-18-1 Frequency of excision Г \*\*\*\* 100 attR attL Chromosome int \_\_\_\_\_ Sp\_2918 10-1 ssrA Rep - cox 10-2 Excision 10-3 attB P2Sp-R 10-4 P2Sp-F GyrB-F GyrB-R attP 10-5 ssrAdel Sp\_2918 10-6 Red cot P2Sp phage circle ΔP2Sp<sup>Ex</sup> chromosome Int 4 WT ∆int ∆rep W3-18-1/pHGE W3-18-1 С Е W3-18-1/pHGE attPlattB (bp) Е Int Cox Rep M 150 Phage circle number \*\*\* \*\*\* 5000 -\*\*\* \*\*\* 3000 -100attB 2000 -1500 -50 5000 -3000 gyrB 2000 -0 1500 cot Reg 4 m WT Aint Arep W3-18-1/pHGE W3-18-1

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### Α W3-18-1 ssrA P2Sp attP W3-18-1 P2Sp excision CCGCCTCCACCA attR ∆P2Sp<sup>Ex</sup> "ticle attB CCGCC-CCACCA attL CCGCCTCCACCA ∆P2Sp<sup>Ex</sup> ∆ssrA С в SsrA (W3-18-1) SsrAdelU (AP2SpEx) W3-18-1 A3' ∆P2Sp<sup>Ex</sup> 5 000000 ACCOCC P2Sn excision ∆ssrA tRNA-like domain Wobble base pair W3-18-1 ∆P2Sp<sup>Ex</sup> T-loop T-loc ∆ssrA 1 V-loop W3-18-1 V-loop mRNA-like ∆P2Sp<sup>Ex</sup> Tag ORF Tag ORF domain **∆**ssrA Tag peptide ANDDNYALAA ANDDNYALAA D Е LB 8 8 W3-18-1 Growth(OD<sub>600</sub>) ∆P2Sp<sup>Ex</sup> -0-Growth(OD<sub>600</sub>) 6 -∆ssrA 4 4 Accepte 2 2 04 0-0 2 4 6 8 10 0 Time (h) Normalized biofilm (OD<sub>540</sub>/OD<sub>620</sub>) **H** G 🛙 W3-18-1 🔲 ΔΡ2Sp<sup>Ex</sup> 🔲 ΔssrA Normalized biofilm (OD<sub>640</sub>/OD<sub>620</sub>) 00 10 70 50 50 1.0 0.8 0.6 0.4 0.2 0.0 24 h 48 h



(41 genes, 34 784 bp) P2Sp

attE

es, 34 784 bp

10-1 10-2 10-3 10-4 10-5 10-6 10-7

SW-LB

W3-18-1

- AssrA

2

4

ΔP2Sp<sup>Ex</sup>/pHGE-ssrA

■ ∆ssrA/pHGE-ssrA

□ AssrA/pHGE-ssrA

24 h

DP2Sp<sup>Ex</sup>/pHGE-ssrA<sup>delU</sup>

6

Time (h)

8

48 h

-O- AP2SpEx

attRSp\_2918 ssrA P2Sp ge

Kanamycin

Gentamycin

N

-

10



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	Lysogeny	->	Induction -)	<ul> <li>Costs (Individual level)</li> </ul>		Benefits (Population level)	
1.1		1 1		For phage	For bacteria	For phage	For bacteria
P2 (W3-18-1)	Alle	SPI		None	Disruption of SsrA Growth inhibition Individual death	P2 reproduction	Increased biofilm Increased expression of transporter system
P4 (MR-1)		Cold induction	Pa	P4 depletion without replication	Disruption of SsrA Growth inhibition	None	Increased biofilm Cold adaption