Deep-sea bacteriophages facilitate host utilization of polysaccharides

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Abstract

Bacteriophages are ubiquitous in nature and play key roles in various ecosystems. They impact microbial community composition and reprogram the metabolism of diverse host cells using auxiliary metabolic genes (AMGs). Whether bacteriophages can reprogram host polysaccharide metabolism through AMGs remains unclear, however. Here, we found for the first time that polysaccharides induce the production of different types of bacteriophages in two deep-sea *Lentisphaerae* strains (WC36 and zth2). Through physiological assays, genomic analysis and transcriptomics assays, we found that these bacteriophages might assist their hosts in metabolizing polysaccharides through AMGs. Moreover, the isolated bacteriophages could effectively assist a marine bacterium (*Pseudomonas stutzeri* 273) in metabolizing and utilizing polysaccharide to promote its growth. These findings shed light on the importance of atypical and poorly understood virus-host interactions and bring us closer to understanding the potential role of deep-sea viruses in marine ecosystems.

Introduction

Viruses are the most abundant and genetically diverse biological entities on this planet (Suttle, 2007), and potentially play a role in shaping microbial abundances, community structure, and the evolutionary trajectory of core metabolisms (Sullivan et al., 2006; Samson et al., 2013; Zimmerman et al., 2020). The effect of bacteriophages (viruses that infect bacteria) on their specific hosts and within the microbial community is largely determined by their lifestyle. Traditionally, most bacteriophage life cycles are classified as being either lytic or lysogenic (Du Toit, 2017). During the lytic cycle, phages hijack the host's metabolic machinery for replication of their own genome and the production of progeny particles that are released through lysis. By contrast, during lysogeny, phages integrate their genomes into the host chromosome (or exist in the extrachromosomal form) and enter a dormant state, with the potential to re-enter a lytic cycle and release progeny by environmental changes at a later stage. Currently, more and more attention has been paid to chronic life cycles where bacterial growth continues despite phage reproduction (Hoffmann Berling and Maze, 1964). During a chronic cycle, progeny phage particles are released from host cells via extrusion or budding without killing the host (Putzrath and Maniloff, 1977; Russel, 1991; Marvin et al., 2014), instead of lysis of the host cell. Chronic infections are common among eukaryotic viruses (Godkin and Smith, 2017), however, only a handful of chronic viruses have been described for prokaryotes (Roux et al., 2019; Alarcón-Schumacher et al., 2022). The best-studied examples of chronic infection are those from filamentous ssDNA (single-stranded DNA) phages that to date have primarily been identified in the order *Tubulavirales*, which currently involves three families (*Inoviridae*, *Paulinoviridae* and *Plectroviridae*) (Ackermann, 2007, 2009; Knezevic et al., 2021; Chevallereau et al., 2022). One of the most distinctive features of inoviruses is their ability to establish a chronic infection whereby their genomes may either integrate into the host chromosome or reside within the cell in a non-active form, and inoviruses particles are continuously released without killing the host. In addition, tailless phages enclosed in lipid membrane are also released from hosts

during chronic life cycles (Liu et al., 2022). Recent studies indicate that chronic cycles are more widespread in nature than previously thought (Mäntynen et al., 2021), and the abundance of inoviruses in ecosystem may have been far underestimated (Roux et al., 2019). These findings have suggested that plenty of novel phages in the biosphere may be produced through chronic life cycles, but whether chronic cycles are associated with specific environments or ecological conditions remains to be thoroughly explored (Chevallereau et al., 2022).

Marine ecosystems always influence the operating conditions for life on earth via microbial interaction networks (Falkowski et al., 1998; Faust and Raes, 2012; Wigington et al., 2016), which are modulated by viruses through impacting their lifespan, gene flow and metabolic outputs (Suttle, 2005). A majority of these viruses are bacteriophages, which exist widely in oceans (Breitbart, 2012). In addition to core viral genes encoding viral structural proteins (Brum et al., 2016), bacteriophages also encode various auxiliary metabolic genes (AMGs) (Brum and Sullivan, 2015) that provide supplemental support to key steps of host metabolism. For instance, AMGs of marine bacteriophages have been predicted to be involved in photosynthesis (Mann et al., 2003), nitrogen- (Ahlgren et al., 2019; Gazitúa et al., 2021), sulfur- (Anantharaman et al., 2014; Roux et al., 2016), and phosphorus cycling (Zeng and Chisholm, 2012), nucleotide metabolism (Sullivan et al., 2005; Dwivedi et al., 2013; Enav et al., 2014) and almost all central carbon metabolisms in host cells (Hurwitz et al., 2013). However, AMGs of chronic phages have not been reported. Thus, it is worth exploring whether chronic phages could carry AMGs and assist host metabolism during chronic infection.

The deep sea harbors abundant and undiscovered viruses, which potentially control the metabolism of microbial hosts and influence biogeochemical cycling (Li et al., 2021). However, the vast majority of deep-sea bacteria cannot be cultivated, which greatly limits the host range of deep-sea bacteriophages, resulting in most unknown bacteriophages could not be isolated in the laboratory. Thus, it is of great significance to further identify unknown phages in the deep sea and explore their relationship with microbial hosts and even marine ecosystems. Here, we report that

polysaccharides can induce deep-sea *Lentisphaerae* bacteria (difficult-to-cultivate microorganisms) to release some bacteriophages. These bacteriophages might assist host metabolism to degrade polysaccharide and thereby promote host growth via AMGs.

Results and Discussion

Polysaccharides promote the growth of deep-sea *Lentisphaerae* strain WC36 and stimulate the expression of phage-associated genes

As a primary carbon source, polysaccharides are a ubiquitous energy source for microorganisms in both terrestrial and marine ecosystems (Zheng et al., 2021a). In our previous work, we successfully isolated a novel *Bacteroidetes* species through a polysaccharide degradation-driven strategy from the deep-sea cold seep (Zheng et al., 2021a). Of note, using the same approach and deep-sea sample replicates, we also cultured a bacterial strain WC36 that was identified as a member of the phylum *Lentisphaerae* by 16S rRNA gene sequence-based phylogenetic analysis (Fig. 1A). As expected, growth assays showed that two kinds of polysaccharides including laminarin and starch could promote strain WC36 growth (Fig. 1B). In particular, supplementation of 10 g/L laminarin and 10 g/L starch in rich medium resulted in ~7-fold and ~4-fold growth promotion respectively compared to cultivation in rich medium (Fig. 1B).

To explore the reasons behind this significant growth promotion by polysaccharide supplementation, we performed a transcriptomic analysis of strain WC36 cultured in rich medium supplemented either with or without 10 g/L laminarin for 5 days and 10 days. In addition to the up-regulation of genes related to glycan transport and degradation, when 10 g/L laminarin was added in the rich medium, the most upregulated genes were phage-associated (e. g. phage integrase, phage portal protein) (Fig. 1C and Supplementary Table 1). Consistently, RT-qPCR results (Fig. 1D) confirmed upregulation of some genes encoding phage-associated proteins, as shown in Fig. 1C. Therefore, we speculate that the metabolism of polysaccharides in strain WC36 might be closely connected with the phage production.

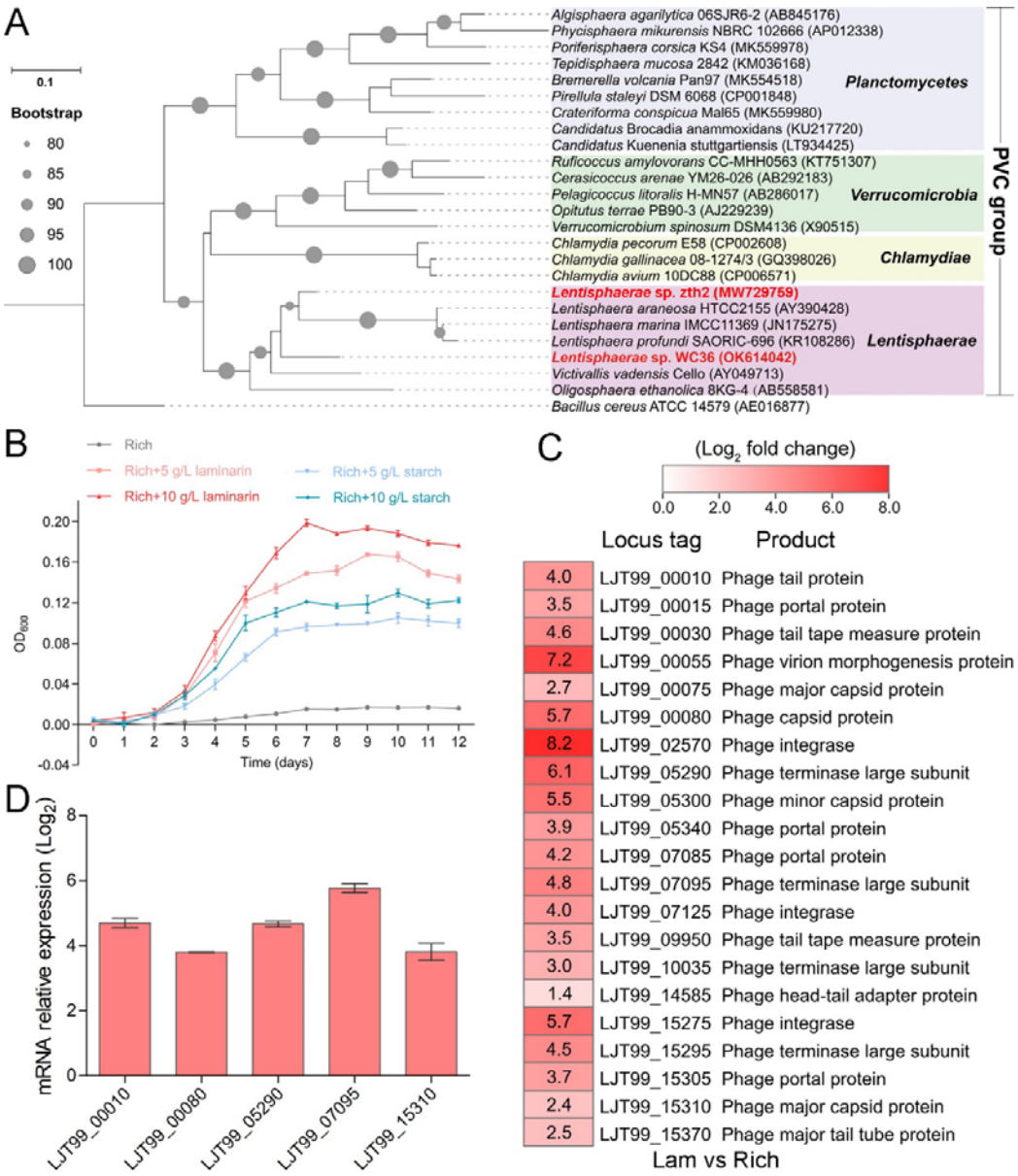

Fig. 1. Polysaccharides promote the deep-sea *Lentisphaerae* strain WC36 growth and stimulate the expression of phage-associated genes. (A) Maximum likelihood phylogenetic tree of 16S rRNA gene sequences from strain WC36, strain zth2, and some *Planctomycetes-Verrucomicrobia-Chlamydia* (PVC) group bacteria. *Bacillus cereus* ATCC 14579 was used as the outgroup. Bootstrap values (%) > 80 are indicated at the base of each node with the gray dots (expressed as percentages of 1000 replications). (B) Growth assays of strain WC36 cultivated in rich medium either without supplementation, with 5 g/L or 10 g/L laminarin or with 5 g/L or 10 g/L starch. (C) Transcriptomics-based heat map showing all up-regulated genes encoding phage-associated proteins in strain WC36 cultured in rich medium supplemented with 10 g/L laminarin. "Rich" indicates strain WC36 cultivated in rich medium; "Lam" indicates strain WC36 cultivated in rich medium supplemented with 10 g/L laminarin. (D) RT-qPCR detection of the expression of some genes encoding phage-associated proteins shown in panel C. The heat map was generated by Heml 1.0.3.3. The numbers in panel C represent multiple differences in gene expression (by taking $\log_2$ values).

Polysaccharides induce the production of bacteriophages in *Lentisphaerae* strain WC36

To test our hypothesis that polysaccharides might induce strain WC36 to release bacteriophages, we grew strain WC36 in rich medium supplemented with or without laminarin or starch and isolated bacteriophages from the cell suspension supernatant according to established standard protocols (Lin et al., 2012). Based on the growth curve of strain WC36, we found that the strictly anaerobic WC36 grew much slower than normal aerobic bacteria. Therefore, different to the typical sampling time (24 h) for bacteriophage isolation from other bacteria (Jiang and Paul, 1996; Weinbauer et al., 1999), we selected a longer sampling time (10 days) to extract bacteriophages. TEM observation showed that many different shapes of phage-like structures indeed existed in the supernatant of WC36 cells cultured in rich medium supplemented with laminarin (Fig. 2A, panels II-IV) or starch (Fig. 2A, panels V-VIII). In contrast, we did not observe any phage-like structures in the supernatant of WC36 cells cultured in rich medium (Fig. 2A, panel I) or in different media without culturing bacterial cells (Supplementary Fig. 1A, B). These results suggest that polysaccharides indeed stimulate the production of various bacteriophages from strain WC36. Correspondingly, in the presence of laminarin, different forms of bacteriophages were observed by TEM, with filamentous ones dominant (Fig. 2A). The length of these filamentous phages was about ~0.4 μm to ~8.0 μm (Fig. 2A, panel II), and the size of the hexagonal phages was less than 100 nm (Fig. 2A, panel IV). In the presence of starch, in addition to filamentous and hexagonal phages (Fig. 2A, panels V, VI, and VIII), we also observed a kind of icosahedral *Microviridae*-like phage with a diameter of 25-30 nm (Fig. 2A, panel VII).

Given that we found bacteriophages in cultures of strain WC36, we next sought to explore whether bacteriophages adhering to bacterial cells could be observed. To this end, we checked the morphology of strain WC36 in the absence or presence of polysaccharides. As expected, with TEM we observed many filamentous phage-like structures around strain WC36 cells cultivated in rich medium supplemented with laminarin (Fig. 2B, panel II) and starch (Fig. 2B, panels III-IV). In contrast, we could

not find any phage-like structures around strain WC36 cells cultivated either in rich medium (Fig. 2B, panel I), in rich medium supplemented with 10 g/L laminarin (Supplementary Fig. 2A) or in 10 g/L starch without culturing bacterial cells (Supplementary Fig. 2B). Moreover, based on TEM observation of ultrathin sections of strain WC36 cultured in medium supplemented with polysaccharide, we even observed filamentous phages that were being released from or entering into host cells via a kind of extrusion or budding structure around bacterial cells (Fig. 2C, panels II-IV), suggesting that filamentous bacteriophages might be released chronically as reported previously (Chevallereau et al., 2022; Liu et al., 2022).

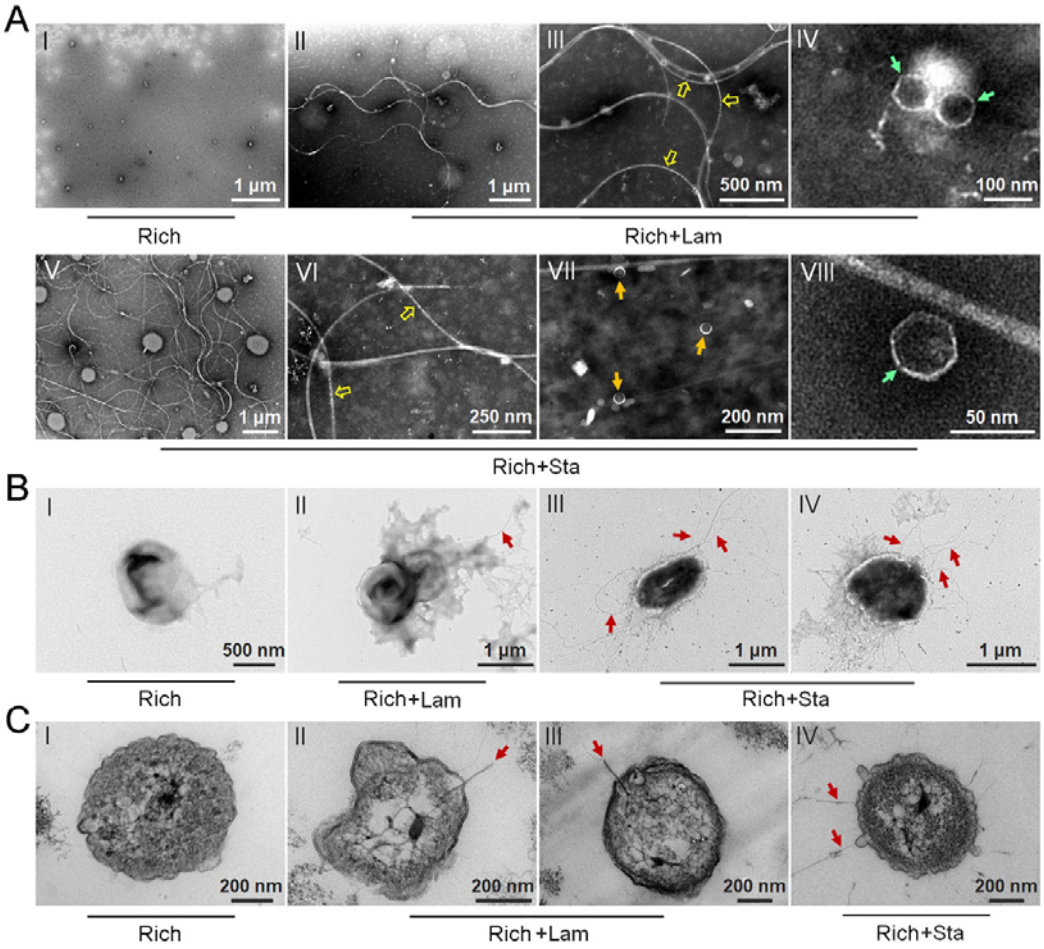

Fig. 2. Polysaccharides induce the production of bacteriophages in *Lentisphaerae* strain WC36. (A) TEM observation of phages extracted from the supernatant of WC36 cells cultured in rich medium supplemented with or without polysaccharide. Panel I shows the absence of phages in the supernatant of WC36 cells cultivated in rich medium. Panels II-IV show the morphology of phages present in the supernatant of WC36 cells cultivated in rich medium supplemented with 10 g/L laminarin. Yellow hollow arrows indicate the typical granular structure of filamentous phages,

and green arrows indicate phage-like particles with other shapes. Panels V-VIII show the morphology of phages present in the supernatant of strain WC36 cells cultivated in rich medium supplemented with 10 g/L starch. Typical filamentous phages are indicated with yellow hollow arrows, and two other kinds of phage-like particles with different shapes are indicated by orange and green arrows, respectively. (B) TEM observation of strain WC36 cultured in rich medium supplemented with or without 10 g/L laminarin or 10 g/L starch. Panel I shows representative morphology of strain WC36 cultivated in rich medium. Panel II shows the morphology of strain WC36 cultivated in rich medium supplemented with 10 g/L laminarin. Panels III-IV show the morphology of strain WC36 cultivated in rich medium supplemented with 10 g/L starch. Red arrows indicate filamentous phages associated with bacterial cells. (C) TEM of an ultrathin section of strain WC36 cultured in rich medium supplemented with or without 10 g/L laminarin or 10 g/L starch. Panel I shows an ultrathin section of strain WC36 cultivated in rich medium; Panels II-III show ultrathin sections of strain WC36 cultivated in rich medium supplemented with 10 g/L laminarin; Panel IV shows an observation of the ultrathin section of strain WC36 cultivated in rich medium supplemented with 10 g/L starch. Red arrows indicate filamentous phages being released from or entering bacterial cells.

To further verify the release style of bacteriophages from strain WC36 in the presence of polysaccharide, we prolonged the incubation time of this strain up to 30 days in medium supplemented with 5 g/L or 10 g/L laminarin. Strain WC36 showed stable growth after it entered the stationary phase (Fig. 3A). The bacterial cells maintained their cell shape intact regardless of the presence of laminarin, indicating their healthy state after for a long time (Supplementary Fig. 3A, panels I-III). Apparently, the replication and release of bacteriophages in strain WC36 did not kill the host cell, consistent with the key feature of chronic bacteriophages (Howard-Varona et al., 2017). With an increase of culture time (5, 10, or 30 days) and laminarin concentration (5 or 10 g/L), the number of released bacteriophages increased (Supplementary Fig. 3A, panels IV-VI; Supplementary Fig. 4, panels IV-IX). Specifically, the average number per microliter of filamentous phages and hexagonal phages extracted from the supernatant of strain WC36 cultured in rich medium supplemented with 10 g/L laminarin for 5, 10, 30 days (around 9.7, 29, 65.3 and 9, 30, 46.7) was higher than those cultured in rich medium supplemented with 5 g/L laminarin for 5, 10, 30 days (around 4.3, 13.7, 35.3 and 4, 11.3, 17.7), respectively (Fig. 3B, C).

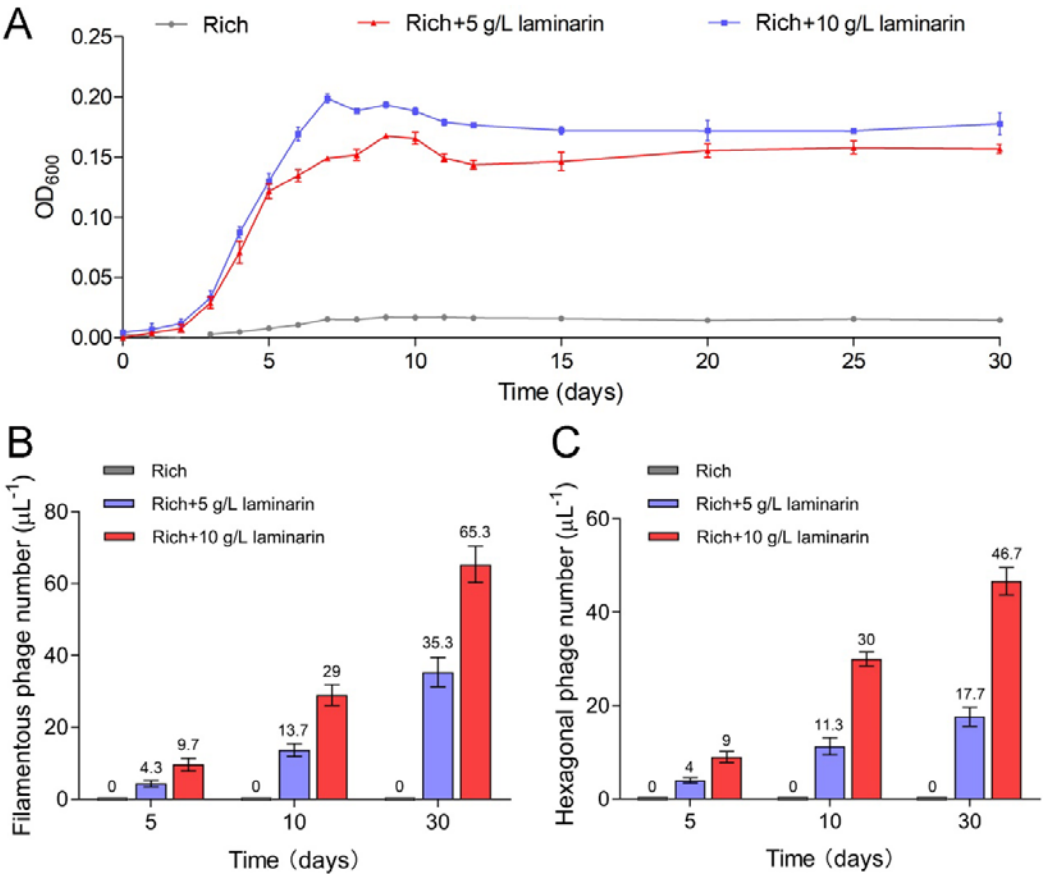

Fig. 3. Bacteriophages induced from *Lentisphaerae* strain WC36 by polysaccharides are released via chronic infections. (A) Growth curve of strain WC36 cultivated in either rich medium alone or rich medium supplemented with 5 g/L or 10 g/L laminarin for 30 days. The number of filamentous phages (B) and hexagonal phages (C) extracted from 1 μL of the supernatant of strain WC36 cell cultured in rich medium supplemented with or without 5 g/L or 10 g/L laminarin (for 5, 10, and 30 days). The average numbers are shown at the top of the bar charts.

In addition, in the presence of laminarin we observed many extrusions or buddings around bacterial cells (Supplementary Fig. 3B, panels II-III), which are typical cellular structures in host cells used for chronic bacteriophage release (Marvin et al., 2014; Krupovic and Consortium, 2018). In contrast, phage-like structures with extrusions or buddings were not found in cells of strain WC36 cultivated in rich medium (Supplementary Fig. 3B, panel I). Hence, these results collectively suggest that filamentous phages induced from deep-sea strain WC36 replicate in a chronic manner. However, the morphology and process of hexagonal phages that entry and exit into the WC36 cells were not observed.

Polysaccharides promote the growth of deep-sea *Lentisphaerae* strain zth2 and induce bacteriophage production

To explore whether the production of bacteriophages induced by polysaccharide is an individual case, we further checked growth promotion and bacteriophage induction by polysaccharide in another cultured deep-sea *Lentisphaerae* - strain zth2 - which was isolated by the same polysaccharide degradation-driven strategy as that used for strain WC36. 16S rRNA gene sequence similarity calculation using the NCBI server indicated that the closest relative of strain zth2 was strain WC36 (90.4%), which was lower than the threshold sequence identity (94.5%) for distinct genera (Yarza et al., 2014). Based on a maximum likelihood tree and similarity of 16S rRNA gene sequences, we propose that strain zth2 and WC36 are the type strains of two different novel genera, both belonging to the *Lentisphaeraceae* family, *Lentisphaerales* order, *Lentisphaeria* class. Consistently, laminarin indeed promoted the growth of strain zth2 (Fig. 4A). Analysis of the differential transcriptome and RTq-PCR of strain zth2 cultured in either unsupplemented medium or with laminarin showed that many genes encoding critical factors associated with the phage life cycle were up-regulated (ranging from 4 to 200 fold) (Fig. 4B, C and Supplementary Table 2), suggesting that phages might also be involved in the utilization of laminarin by strain zth2, the same as in strain WC36.

To further confirm the existence of bacteriophages in the supernatant of strain zth2 cell suspension cultured in medium supplemented with polysaccharide, we performed bacteriophage isolation from the supernatant of strain zth2 cultures followed by TEM. Indeed, many filamentous-, hexagonal- and icosahedral phages were clearly observed in the culture of strain zth2 grown in the presence of laminarin (Fig. 4D, panels I-III) and starch (Fig. 4D, panels IV-V), but not in rich medium short of polysaccharide (Fig. 4D, panel VI). This suggests that bacteriophages were induced by supplementation of polysaccharide to strain zth2.

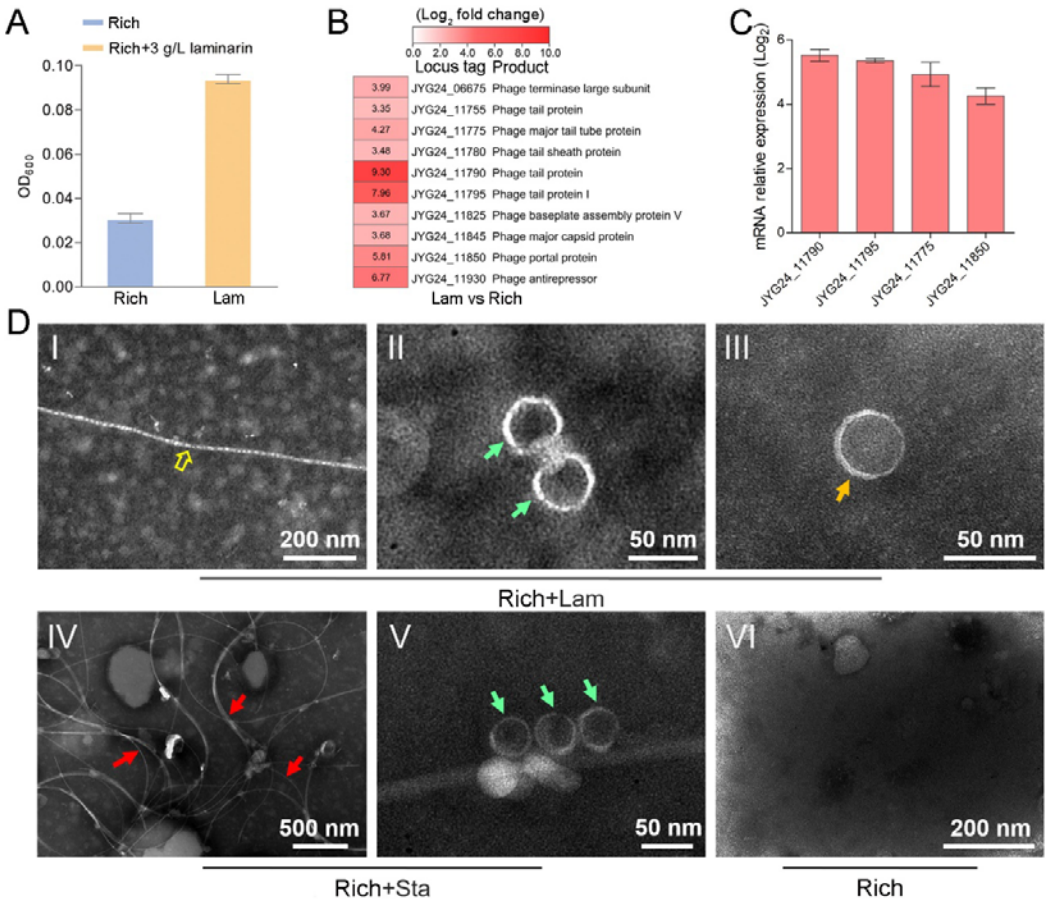

Fig. 4. Polysaccharides promote the growth of deep-sea *Lentisphaerae* strain zth2 and induce the production of bacteriophages. (A) Growth assays of strain zth2 cultivated in rich medium supplemented with or without 3 g/L laminarin for four days. (B) Transcriptomics-based heat map showing all up-regulated genes encoding phage-associated proteins in strain zth2 cultured in rich medium supplemented with 3 g/L laminarin. (C) RT-qPCR detection of the expressions of some genes encoding phage-associated proteins shown in panel B. The numbers in panel C represent multiple differences in gene expression (by taking $\log_2$ values). (D) TEM observation of phages extracted from the supernatant of a cell suspension of strain zth2 cultured in the rich medium supplemented with or without polysaccharides. Panels I-III show the morphology of phages in the cell supernatant of strain zth2 cultivated in rich medium supplemented with 3 g/L laminarin. Typical filamentous phage is indicated with yellow hollow arrows, and two other kinds of phage-like particles with different shapes are indicated by orange and green arrows, respectively. Panels IV-V show the morphology of phages present in the cell supernatant of strain zth2 cultivated in rich medium supplemented with 3 g/L starch. Typical filamentous phages are indicated with red arrows, and other phage-like particles with different shapes are indicated by green arrows. Panel VI shows that no phages were observed in the cell supernatant of strain zth2 cultivated in rich medium. “Rich” indicates strain zth2 cultivated in rich medium; “Lam” indicates strain zth2 cultivated in rich medium supplemented with 3 g/L laminarin.

In addition, transcriptome and RT-qPCR results showed that genes encoding bacterial secretion system-related proteins were up-regulated in strains WC36

(Supplementary Fig. 5A, B) and zth2 (Supplementary Fig. 5C, D) cultured in medium supplemented with laminarin. It has been reported that filamentous phages utilise host cell secretion systems for their own egress from bacterial cells (Davis et al., 2000; Bille et al., 2005). Therefore, the filamentous phages induced from strains WC36 and zth2 might also get in and out of host cells using bacterial secretion systems. Taken together, we conclude that laminarin effectively promotes the growth of deep-sea *Lentisphaerae* strains WC36 and zth2, and induces the production of bacteriophages. Distinct from previous reports that bacteriophages are induced under traditional conditions such as low temperature (Wang et al., 2007), mitomycin C (Mazaheri Nezhad Fard et al., 2010), and ultraviolet irradiation (McKay and Baldwin, 1973), here, we report that polysaccharides induce the production of bacteriophages.

Bacteriophages reprogram the polysaccharide metabolism of *Lentisphaerae* strains WC36 and zth2

In natural ecosystems, viral infections indirectly influence microbial metabolic fluxes, metabolic reprogramming, and energy homeostasis of host cells (Howard-Varona et al., 2020). For example, cyanoviruses possess AMGs encoding core photosynthetic reaction centers (Sullivan et al., 2006) which are highly expressed during infection, thus stimulating photosynthesis and enhancing viral abundance (Lindell et al., 2007). In this study, we clearly show that polysaccharide effectively promotes bacterial growth and simultaneously induces the production of bacteriophages, suggesting a close relationship between bacteriophages and host polysaccharide metabolism. Therefore, we sought to ask whether bacteriophages could reprogram polysaccharide metabolism in deep-sea bacteria. For this purpose, we sequenced the genomes of the bacteriophages induced by polysaccharides in *Lentisphaerae* strains WC36 and zth2. Eventually, two assembled genomes (Phage-WC36-1, 8.0 kb; Phage-WC36-2, 28.3 kb) were obtained from the bacteriophages of strain WC36 (Fig. 5A, B and Supplementary Tables 3, 4). Meanwhile, two assembled genomes (Phage-zth2-1, 8.0 kb; Phage-zth2-2, 40.4 kb) were obtained from the phages of strain zth2 (Fig. 5A, C and Supplementary Tables 3, 5). Subsequently, we carefully compared the

bacteriophage genomes with those of the corresponding hosts (strains WC36 and zth2) using Galaxy Version 2.6.0 (https://galaxy.pasteur.fr/) (Afgan et al., 2018) with the NCBI BLASTN method and used BWA-mem software for read mapping from host whole genome sequencing (WGS) to these bacteriophages. These analyses both showed that the bacteriophage genomes are completely outside of the host chromosomes, as reported previously (Chevallereau et al., 2022). Through comparison of genome sequence (Supplementary Fig. 6), IMG database analysis, and phylogenetic analysis (Supplementary Fig. 7), we confirmed that Phage-WC36-1 was the same as Phage-zth2-1 (inoviruses, belonging to the family *Inoviridae*, order *Tubulavirales*, class *Faserviricetes*), indicating that inoviruses might be common in the *Lentisphaerae* phylum. Inovirus members were the dominant population in the active group of viruses from deep-sea sediments, and encode some proteins contributing to host phenotypes and ultimately alter the fitness and other behaviors of their hosts (Engelhardt et al., 2015; Mai-Prochnow et al., 2015). A maximum likelihood tree showed that both Phage-WC36-2 and Phage-zth2-2 belonged to the class *Caudoviricetes* (Supplementary Fig. 8). In addition to genes encoding phage-associated proteins within the assembled bacteriophage genomes, there are also many genes encoding proteins associated with polysaccharide transport and degradation. These polysaccharide metabolizing proteins include glycoside hydrolase family 18 protein (GH18, HMM e-value 1.5e-14) (Fig. 5A), TonB-system energizer ExbB (Koebnik, 2005), O-polysaccharide acetyltransferase (Lunin et al., 2020), glucosaminidase, glycoside hydrolase family 127 protein (best BLASTP hit e-value 3e-25) (Fig. 5B) and transglycosylase (Fig. 5C). These CAZymes (GH18 and GH127) are specifically responsible for degrading polysaccharides (Cantarel et al., 2009). Energizer ExbB is an important part of the TonB-system, which is often present in *Bacteroides* and responsible for transport of oligosaccharides from the outer membrane into the periplasm (Foley et al., 2016). It is absent in the strain WC36 and zth2 genomes, however. Therefore, these AMGs encoding TonB-system energizer ExbB and glycoside hydrolases might reprogram the polysaccharide metabolism of host cells (strains WC36 and zth2) by providing supplemental support to key

metabolic processes. Overall, bacteriophages induced by polysaccharides contain specific AMGs associated with polysaccharide degradation, which may play a key role in the degradation and utilization of polysaccharide by host cells.

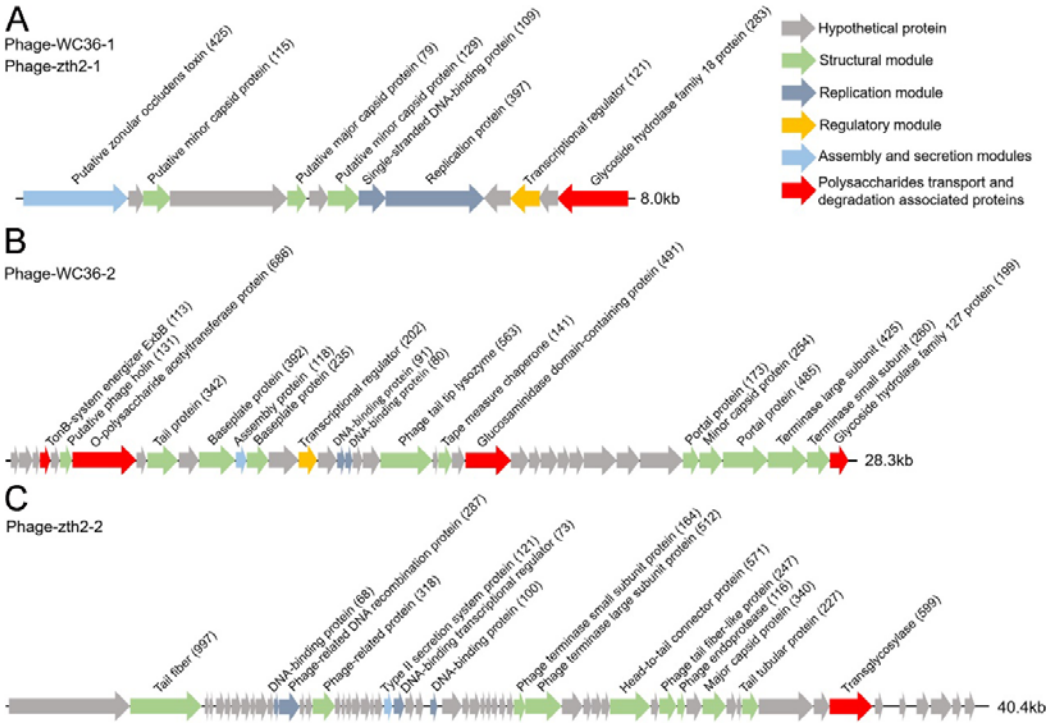

Fig. 5. Genomic organization of bacteriophages released from *Lentisphaerae* strains WC36 and zth2 cultured in rich medium supplemented with polysaccharide. (A) Schematic of the genomic composition of Phage-WC36-1 and Phage-zth2-1. (B) A diagram showing the genomic composition of Phage-WC36-2. (C) The genomic composition of Phage-zth2-2. Arrows represent different ORFs and the direction of transcription. The main putative gene products of the phages are shown, and the numbers in brackets indicate the numbers of amino acids within each ORF. Hypothetical proteins are indicated with gray arrows, structural modules are indicated by green arrows, the replication module is indicated by blue-grey arrows, the regulatory module is indicated by golden arrows, the assembly and secretion modules are indicated by blue arrows and auxiliary metabolic genes (AMGs) encoding proteins associated with polysaccharide transport and degradation are indicated by red arrows. The size of the phage genomes is shown behind each gene cluster.

Bacteriophages released from *Lentisphaerae* strain WC36 promote the growth and polysaccharide metabolism of a marine bacterium, *Pseudomonas stutzeri* 273

Given that the bacteriophages released from *Lentisphaerae* strain WC36 (named Phages-WC36 in this study) could reprogram bacterial polysaccharide metabolism and thereby promote host growth, we next sought to determine the capability of these

bacteriophages to reprogram polysaccharide metabolism and promote growth in other marine bacteria. To this end, we chose a marine bacterium - *Pseudomonas stutzeri* 273 (Wu et al., 2016) - and tested the potential growth promotion driven by Phages-WC36. Growth assays showed that Phages-WC36 could promote strain 273 growth by assisting its metabolism and utilization of starch (Fig. 6A). In particular, supplementation with Phages-WC36 and 5 g/L starch in basal medium resulted in 6~10-fold growth promotion compared to basal medium without Phages-WC36 or starch (Fig. 6A). In addition, we observed many filamentous phage and hexagonal phage structures in the supernatant of strain 273 cultivated in basal medium supplemented with 5 g/L starch and Phages-WC36 for 24 h. In contrast, we could not find any phage-like structures in three other types of media. Specifically, the average number of filamentous phages and hexagonal phages extracted from the supernatant of strain 273 cultured in basal medium supplemented with 5 g/L starch and Phages-WC36 (~44.7 and ~29, respectively) was higher than that in three other media types (where it was close to zero) (Fig. 6B). To test whether Phages-WC36 could infect strain 273, the strain 273 cultivated in basal medium supplemented with 5 g/L starch and Phages-WC36 was inoculated in basal medium and basal medium supplemented with 5 g/L starch for three consecutive times (Fig. 6C). After 3 passages of serial cultivation in basal medium supplemented with 5 g/L starch, we found that filamentous phages (~45.3, Fig. 6D) and hexagonal phages (~21.7, Fig. 6E) were also present in basal medium supplemented with starch, but not in the basal medium, which may mean that Phages-WC36 could infect strain 273 and starch is an important inducer. Consistently, genes encoding bacterial type II/IV/VI secretion system-related proteins were up-regulated in strain 273 cultured in basal medium supplemented with 5 g/L starch and 20 μl/mL Phages-WC36 in comparison to basal medium with 5 g/L starch (Supplementary Fig. 9). Upregulation of these genes may help filamentous phages get in and out of host cells (Davis et al., 2000; Bille et al., 2005). Moreover, the expression of genes encoding the TonB-dependent receptor and CAZymes was upregulated in strain 273 cultured in basal medium supplemented with starch and Phages-WC36 in comparison to basal medium with starch (Supplementary

Fig. 10A and Supplementary Table 6) or Phages-WC36 (Supplementary Fig. 10B and Supplementary Table 7). These results suggest that Phages-WC36 proliferate in strain 273 and promote growth by assisting in starch metabolism and utilization. Consequently, we propose that Phages-WC36 might infect other hosts and assist them in degradation and utilization of polysaccharides. This provides stronger evidence that the relationship between Phages-WC36 and their hosts is not a fight to the death between enemies, but in contrast a mutualistic relationship between partners (Shkoporov et al., 2022). Most deep-sea microbes are anaerobic, which limits investigation of bacteriophages in their original host. *P. stutzeri* 273 is a typical aerobic bacterium, and thus our study provides an effective way to replicate deep-sea bacteriophages and perform functional analysis in aerobic heterologous hosts.

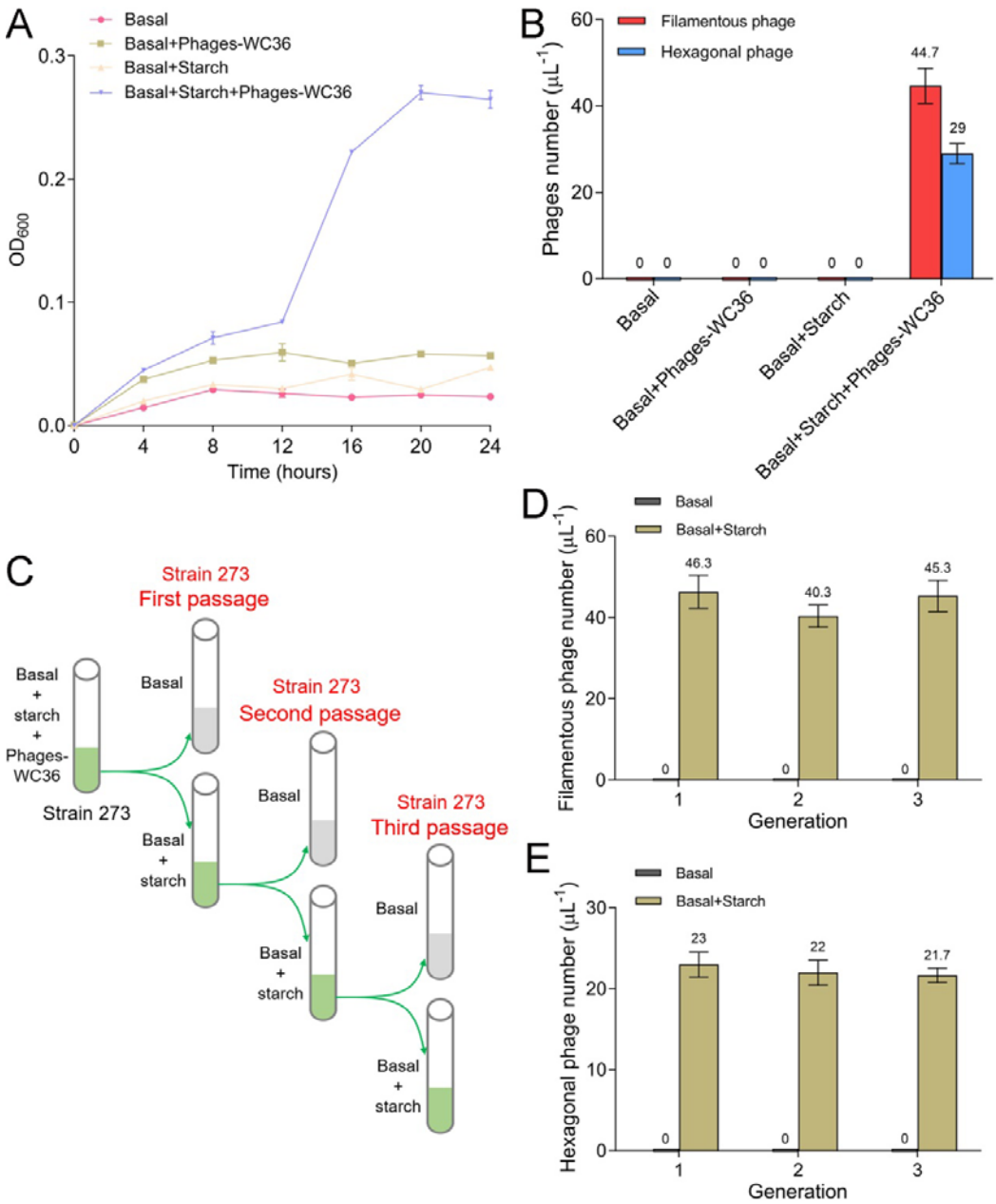

Fig. 6. Bacteriophages released from *Lentisphaerae* strain WC36 promote the growth and polysaccharide metabolism of a marine bacterium *Pseudomonas stutzeri* 273. (A) Growth curve and status of *P. stutzeri* 273 cultivated in basal medium, basal medium supplemented with 20 μl/mL Phages-WC36, basal medium supplemented with 5 g/L starch, basal medium supplemented with 5 g/L starch and 20 μl/mL Phages-WC36. (B) Number of filamentous phages and hexagonal phages extracted from 1 μL of *P. stutzeri* 273 cell supernatant after culture in four different types of media as in panel A. The average numbers are shown at the top of the bar charts. (C) Continuous culture model of Phages-WC36 infected strain 273. The number of filamentous phages (D) and hexagonal phages (E) extracted from 1 μL of *P. stutzeri* 273 cell supernatant after three successive cultures. The average numbers are shown at the top of the bar charts.

In summary, we demonstrate for the first time that polysaccharides induce the production of bacteriophages in *Lentisphaerae* strains WC36 and zth2, and these bacteriophages contain AMGs encoding TonB-system energizer ExbB and CAZymes that assist host metabolism and utilization of polysaccharide, thereby promoting host growth. We also demonstrate that Phages-WC36 could infect and assist a marine strain 273 to utilize polysaccharides, further expanding our understanding of the significant role of deep-sea bacteriophages. Moreover, the study of deep-sea bacteriophages has the potential to provide insight into the molecular evolution of virus-host interactions in prokaryotes.

Materials and methods

Samples, media, and cultivation conditions

Deep-sea samples used for bacteria isolation in this study were collected from a typical cold seep (E119º17'07.322", N22º06'58.598") at a depth of 1146 m by *RV KEXUE* in July of 2018. Inorganic medium (including 1.0 g/L NH_4Cl, 1.0 g/L $NaHCO_3$, 1.0 g/L CH_3COONa, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.7 g/L cysteine hydrochloride, 500 µl/L 0.1 % (w/v) resazurin, 1 L filtered seawater, pH 7.0) supplemented with 1.0 g/L of different polysaccharides (laminarin, fucoidan, starch, and alginate) under a 100% N_2 atmosphere was used to enrich microbes at 28 °C for one month (Zheng et al., 2021a). The detailed isolation and purification steps were performed as described previously (Zheng et al., 2021b). After pure cultures of deep-sea bacteria were obtained, we cultured them in a rich medium (containing 1.0 g/L peptone, 5.0 g/L yeast extract, 1.0 g/L NH_4Cl, 1.0 g/L $NaHCO_3$, 1.0 g/L CH_3COONa, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.7 g/L cysteine hydrochloride, 500 µl/L 0.1 % (w/v) resazurin, 1 L filtered seawater, pH 7.0) or rich medium supplemented with different polysaccharides for the future research.

Phylogenetic analysis

The 16S rRNA gene tree was constructed based on full-length 16S rRNA gene sequences by the maximum likelihood method. The full-length 16S rRNA gene

sequences of *Lentisphaerae* strains WC36 and zth2 were obtained from their genomes, and the 16S rRNA gene sequences of other related taxa used for phylogenetic analysis were obtained from NCBI (www.ncbi.nlm.nih.gov/). The maximum likelihood phylogenetic trees of Phage-WC36-1 were constructed based on the zona occludens toxin (Zot) and single-stranded DNA-binding protein. The maximum likelihood phylogenetic tree of Phage-WC36-2 and Phage-zth2-2 was constructed based on the terminase large subunit protein (*terL*). These proteins used to construct the phylogenetic trees were all obtained from the NCBI databases. All the sequences were aligned by MAFFT version 7 (Katoh et al., 2019) and manually corrected. The phylogenetic trees were constructed using the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at) (Trifinopoulos et al., 2016). Finally, we used the online tool Interactive Tree of Life (iTOL v5) (Letunic and Bork, 2021) to edit the tree.

Growth assay and transcriptomic analysis of *Lentisphaerae* strains WC36 and zth2 cultured in medium supplemented with laminarin

To assess the effect of polysaccharide on the growth of strain WC36, 100 µl of freshly incubated cells were inoculated in a 15 mL Hungate tube containing 10 mL rich medium supplemented with or without 5 g/L or 10 g/L laminarin (or starch). To assess the effect of laminarin on the growth of strain zth2, 100 µL of freshly incubated cells were inoculated in a 15 mL Hungate tube containing 10 mL rich medium supplemented with or without 3 g/L laminarin. Each condition was performed in three replicates. All the Hungate tubes were incubated at 28 °C. The bacterial growth status was monitored by measuring the OD_{600} value via a microplate reader (Infinite M1000 Pro; Tecan, Mannedorf, Switzerland) every day until cell growth reached the stationary phase.

For transcriptomic analysis, a cell suspension of strain WC36 was harvested after culture in 1.5 L rich medium either without supplementation or with 10 g/L laminarin for 5 days and 10 days. A cell suspension of strain zth2 was harvested after culture in rich medium either without supplementation or with 3 g/L laminarin for 4 days.

Thereafter, these collected samples were used for transcriptomic analysis by Novogene (Tianjin, China). The detailed procedures of the transcriptomic assays including library preparation, clustering, and sequencing, and data analyses are described in the supplementary information.

Bacteriophages isolation

Phage isolation was as described previously with some modifications (Yamamoto et al., 1970; Tseng et al., 1990; Kim and Blaschek, 1991). To induce the production of bacteriophages, strain WC36 was inoculated in rich medium supplemented with or without 10 g/L laminarin or 10 g/L starch, and strain zth2 was cultured in rich medium supplemented with or without 3 g/L laminarin or 3 g/L starch. After 10 days incubation, 60 mL of the different cultures was respectively collected and cells were removed by centrifugation at 8,000 × *g*, 4 °C for 20 minutes three times. The supernatant was filtered through a 0.22 μm millipore filter (Pall Supor, New York, America) and subsequently 1 M NaCl was added to lyse the bacteria and separate phage particles from these bacterial fragments. Then the supernatant was filtered through a 0.22 μm millipore filter and collected by centrifugation at 8,000 × *g*, 4 °C for 20 minutes, and phage particles were immediately precipitated with 100 g/L polyethylene glycol (PEG8000) at 4 °C for 6 hours, and collected by centrifugation at 10,000 × *g*, 4 °C for 20 minutes. The resulting phage particles were suspended in 2 mL SM buffer (0.01% gelatin, 50 mM Tris-HCl, 100 mM NaCl, and 10 mM $MgSO_4$), then the suspension was extracted three times with an equal volume of chloroform (Lin et al., 2012) and collected by centrifugation at 4,000 × *g*, 4 °C for 20 minutes. Finally, clean phage particles were obtained.

Transmission electron microscopy (TEM)

To observe the morphology of bacteriophages, 10 μL phage virions were allowed to adsorb onto a copper grid for 20 minutes, and then stained with phosphotungstic acid for 30 seconds. Next, micrographs were taken with TEM (HT7700, Hitachi, Japan) with a JEOL JEM 12000 EX (equipped with a field emission gun) at 100 kV.

To observe the morphology of strain WC36, cell suspension in rich medium supplemented with or without polysaccharide was centrifuged at 5,000 × *g* for 10 minutes to obtain cell pellets. Subsequently, one part of the cell collection was adsorbed to the copper grids for 20 minutes, then washed with 10 mM phosphate buffer solution (PBS, pH 7.4) for 10 minutes and dried at room temperature for TEM observation. Another part was first preserved in 2.5% glutaraldehyde for 24 hours at 4 °C, and then washed three times with PBS and dehydrated in ethanol solutions of 30%, 50%, 70%, 90%, and 100% for 10 minutes each time. Samples were further fixed in 1% osmium tetroxide for 2 hours and embedded in plastic resin (Zechmann and Zellnig, 2009; Fortunato et al., 2016). Thereafter, an ultramicrotome (Leica EM UC7, Germany) was used to prepare ultrathin sections (50 nm) of cells, and the obtained sections were stained with uranyl acetate and lead citrate (Graham and Orenstein, 2007; Panphut et al., 2011). Finally, all samples were observed with the JEOL JEM-1200 electron microscope as described above. In parallel, the filamentous phage' number in each condition was respectively calculated.

Genome sequencing of bacteriophages

To sequence the genomes of bacteriophages, phage genomic DNA was extracted from different purified phage particles. Briefly, 1 μg/mL DNase I and RNase A were added to the concentrated phage solution for nucleic acid digestion overnight at 37 °C. The digestion treatment was inactivated at 80 °C for 15 minutes, followed by extraction with a Viral DNA Kit (Omega Bio-tek, USA) according to the manufacturer's instructions. Then, genome sequencing was performed by Biozeron Biological Technology Co. Ltd (Shanghai, China). The detailed process of library construction, sequencing, genome assembly and annotation is described in the supplementary information. In order to detect whether these bacteriophage genomes were located within or outside of the host chromosomes, we used the new alignment algorithm BWA-MEM (version 0.7.15) (Li, 2013) with default parameters to perform read mapping of host WGS to these phages. In addition, we also evaluated the assembly graph underlying the host consensus assemblies. Clean reads were mapped to the

bacterial complete genome sequences by Bowtie 2 (version 2.5.0) (Langmead and Salzberg, 2012), BWA (version 0.7.8) (Li and Durbin, 2009), and SAMTOOLS (version 0.1.18) (Li et al., 2009) with the default parameters.

Growth assay of *Pseudomonas stutzeri* 273 cultured in basal medium supplemented with starch and Phages-WC36

In order to verify whether the bacteriophages induced from strain WC36 were able to assist host metabolism and utilization of polysaccharides, we used *Pseudomonas stutzeri* 273 isolated from deep-sea sediment (Wu et al., 2016). Specifically, 50 μL freshly incubated cells (*P. stutzeri* 273) were inoculated in 5 mL basal medium (10 g/L NaCl, 1 L sterilized distilled water), basal medium supplemented with 20 μl/L Phages-WC36, basal medium supplemented with 5 g/L starch, basal medium supplemented with 5 g/L starch and 20 μl/L Phages-WC36 for 24 hours at 28 °C, respectively. The Phages-WC36 used in this growth assay were purified multiple times and eventually suspended in SM buffer. Each condition was performed with three replicates. Bacterial growth was monitored by measuring the OD_{600} value via a microplate reader (Infinite M1000 Pro; Tecan, Mannedorf, Switzerland) every 4 hours until cell growth reached the stationary phase. In addition, phages were extracted from the supernatants of the above four media and their morphology was observed by TEM. In parallel, filamentous phage and hexagonal phage numbers in each condition was respectively calculated by randomly selecting TEM photos.

For transcriptomic analysis, we used cell suspensions of *P. stutzeri* 273 cultured in basal medium supplemented with 20 μl/L Phages-WC36, basal medium supplemented with 5 g/L starch, basal medium supplemented with 5 g/L starch and 20 μl/L Phages-WC36 for 24 hours at 28 °C. These cultures were harvested and sent to Novogene (Tianjin, China) for transcriptome sequencing analysis. The detailed procedures are described in the supplementary information.

Data availability

The complete genome sequences of strains WC36 and zth2 presented in this study

have been deposited in the GenBank database with accession numbers CP085689 and CP071032, respectively. The whole 16S rRNA gene sequences of strains WC36 and zth2 have been deposited in the GenBank database with accession numbers OK614042 and MW729759, respectively. The genome sequences of Phage-WC36-1 (Phage-zth2-1), Phage-WC36-2, and Phage-zth2-2 have been deposited in the GenBank database with accession numbers ON698679, OL791266, and OL791268, respectively. The raw sequencing reads from the transcriptomics analysis have been deposited to the NCBI Short Read Archive (accession numbers: PRJNA946146, PRJNA756144, and PRJNA865570).

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Author contributions

CW, RZ, and CS conceived and designed the study; CW and RZ conducted most of the experiments; TZ helped to isolate the *Lentisphaerae* strain zth2; CW and CS led the writing of the manuscript; all authors contributed to and reviewed the manuscript.

Conflict of interest

The authors declare no competing interests.

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