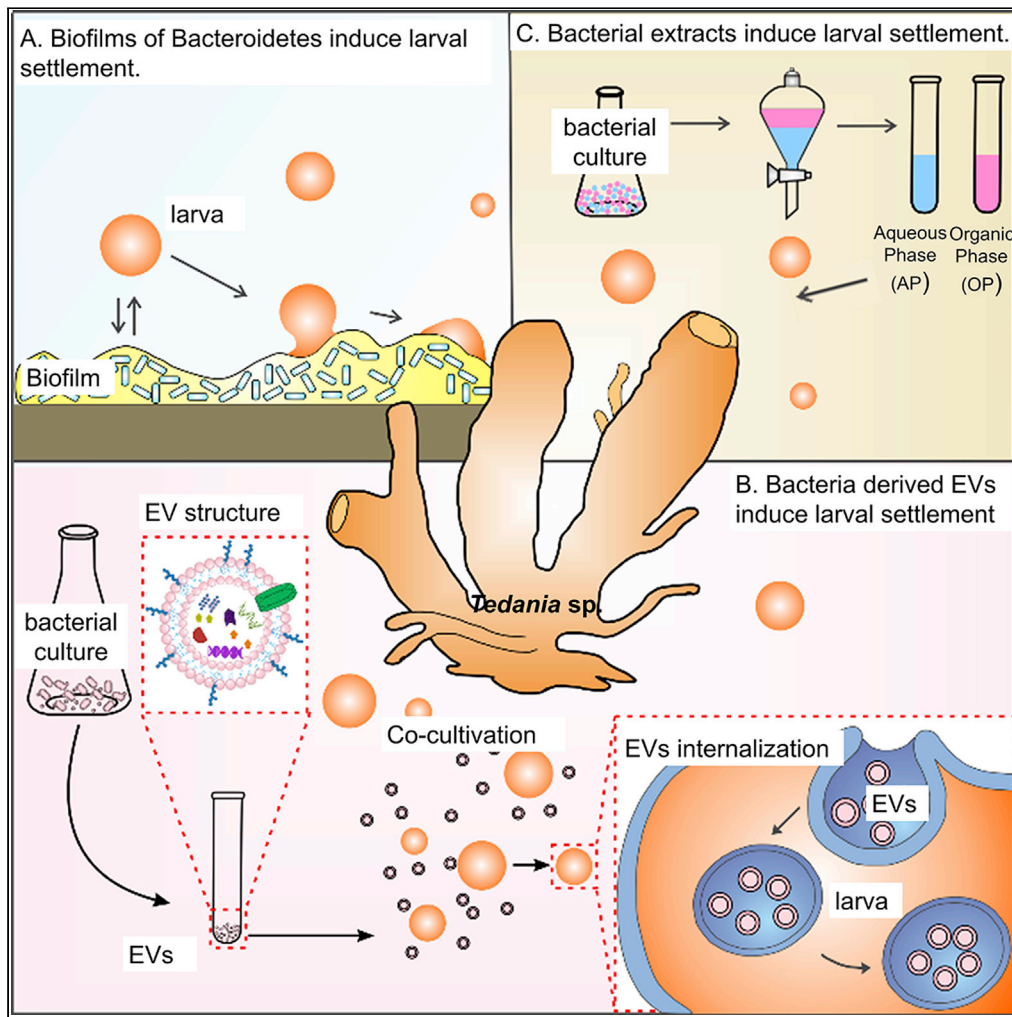


Article

Bacteroidetes bacteria, important players in the marine sponge larval development process



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Highlights

Bacteroidetes strains positively affected sponge *Tedania sp.* settlement

Bacteroidetes strains forming three strategies interacted with sponge

Bacteroidetes extracellular vesicles (EVs) can promote sponge larval settlement

Bacteroidetes EVs were internalized by sponge larva and migrated inside it



Article

Bacteroidetes bacteria, important players in the marine sponge larval development process

Mingyu Li,² Kai Wang,² Chenzheng Jia,² Tan Liu,² Shuo Yang,² Huilong Ou,² and Jing Zhao^{1,2,3,*}

SUMMARY

Bacteroidetes bacteria are frequently found in association with sponges, but their roles in host development are poorly understood. In this study, thirteen bacterial species (12 genera) isolated from the sponge *Tedania* sp. revealed a common ability to significantly promote sponge larval settlement at rates 30.00–53.33% higher than controls ($p < 0.05$). Three effective strategies were adapted: (i) two strains formed biofilms enhancing the settlement rate to 56.67–63.33% within three days. (ii) Five strains secreted hydrosoluble molecules improving larval settlement, reaching 59.17%. (iii) Six species produced extracellular vesicles (EVs) that significantly improved settlement by up to 86.67% ($p < 0.05$). The EV fluorescence demonstrated that they migrated inside the sponge larvae from the planktonic to metamorphosis stage. Generally, marine sponges specifically enrich Bacteroidetes bacteria because of the important player in host development, establishing the basis for reciprocal adaptive co-evolution between the microbial community and animals, even including higher organisms.

INTRODUCTION

Ongoing advances in analytical approaches have revealed a ubiquitous, diverse, and complex bacterial world shared by most living organisms (McFall-Ngai et al., 2013). These microorganisms integrate into the broader ecosystem, generating questions about evolution, symbiosis, and structure (Raina et al., 2018). Biologists have long appreciated the fact that the host-microbe association and coevolution have shaped aspects of life on earth, including the evolution of the eukaryotic cell, multicellularity, and the development of organ systems. In marine environments, invertebrates can house microbial communities as complex and dynamic as that of the human gut (O'Brien et al., 2019). Marine sponges (Phylum Porifera) represent one of the oldest extant metazoans, and sponges distribute globally in all aquatic habitats. Due to sponges' immense filter feeding capacity, numerous microorganisms colonize the tissue, comprising up to 40–60% of the sponge biomass (Pita et al., 2018). The microbial communities of sponge are as diverse and complex as corals but could be far more stable in space and time due to the vertical transmission of bacteria and the requirement of host for special bacteria from surrounding seawater (Erwin et al., 2012; O'Brien et al., 2019). Sponges are increasingly recognized as one of the classic cases of co-divergence of host and microbe (Lucia et al., 2016). The prevalence of these associations implies that sponges can no longer be considered as autonomous entities, and sponges provide evidence that microbial partners contribute to animal biology from individual development to systems ecology (Pita et al., 2018). Microbes take on many roles in sponge nutrition, predation, metabolism, and immunity, but to date, microbes have been rarely studied regarding host larval development. It will be important to learn whether intercellular cohesion in sponges involves bacteria to further understand how associations among bacteria and animals first evolved (McFall-Ngai et al., 2013).

Sponges have ancient and conserved developmental processes, from a free-swimming phase to settlement and metamorphosis. The initiation of settlement and metamorphosis is irreversible which experiences dramatic cell reorganization and delamination (Wanninger, 2015). The successful settlement to optimal habitats is largely linked to the distribution, growth, and survival of marine invertebrates (Steve et al., 2015). Traditionally, animal development has been viewed as an autonomous process directed by the genome. However, sponges both originated and evolved in a microbe-rich marine environment, where

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development, at least in part, was an orchestration involving the animal encoded ontogeny and microbial-host interdomain communication (Alegado et al., 2012; Davidowitz, 2009). From low unicellular protozoa to higher vertebrates, diverse animals have been confirmed to respond to bacterial signals as triggers for morphogenesis or developmental behavior (e.g., larval settlement) (McFall-Ngai et al., 2013).

Although biofilm- or bacteria-induced settlement has been shown for larvae of sponges (Freckelton et al., 2017), more attention has been focused on one single bacterial strain acting on the host instead of one special bacterial genus, family, order, or phyla. The fact was ignored that one bacterial species was more influenced and changed by location, seasons, and host taxon than one bacterial group under the same taxonomy level. Due to marine sponge hosts harboring diverse and complex microbial communities, it is difficult to identify important symbionts and to understand how these communities are structured and how they function. Our previous study showed that members of the phylum Bacteroidetes could be recruited in sponge development, functioning as competent larval-stage-specific associates, where a small, albeit significant amount of bacteria possibly functioned in triggering larval settlement during the sponge life cycle (Wu et al., 2018). Bacteroidetes is a widespread and important phylum for organisms of different developmental dimensions; the bacteria regulate morphogenetic processes in primitive lineages from unicellular organisms to vertebrates (Woznica and King, 2018). In one of the closest living relatives of animals, the choanoflagellate *Salpingoeca rosetta*, the rosette colony development is induced by Bacteroidetes species (Alegado et al., 2012). Many insects host a distinct clade of bacterial symbionts belonging to the phylum Bacteroidetes, and symbiont phylogeny is completely congruent with the phylogeny of insect hosts as far as currently known (Moran et al., 2005). In humans, 90% of microorganisms present in the gut belong to Firmicutes and Bacteroidetes, among which *Bacteroides thetaiotaomicron* increases goblet cell differentiation (Wrzosek et al., 2013). This complexity of one bacterial phylum has also made it challenging to define microbial function and to unravel the myriad host-bacteria interactions.

Phylum Bacteroidetes is global distributed and abundant in oceans only second to Proteobacteria and Cyanobacteria (Fernández-Gómez et al., 2013). In this study, 13 Bacteroidetes species belonging to 12 genera were isolated from the sponge *Tedania* sp. that was dominant along the southeast coast of China. The bacteria were verified to apply different strategies to promote sponge larval settlement. It seems intriguing that coevolution could involve the reciprocal adaptation of one lineage in response to another or could occur through the interaction between a host and its beneficial symbionts (O'Brien et al., 2019). Enhanced knowledge of the interactions between marine invertebrates and associated microbial communities is urgently required, as marine reef ecosystems are facing unprecedented pressures, while manipulation of the microbiome is proposed to be one active way to improve restoration (Glasl et al., 2019). In addition, confirming an intimate association between bacteria and low-complexity marine invertebrates could be beneficial to understanding host-microbe interactions and coevolution of higher-level systems, even in humans, based on shared metabolic functions.

RESULTS

The effect of associated Bacteroidetes strains on the settlement of marine sponges

A total of 13 associated bacterial species were isolated from the sponge *Tedania* sp. and identified to cluster within 12 genera and three main classes of the phylum Bacteroidetes in marine environment (Figure 1A). After co-cultivating with sponge larvae for 8–20 h, 12 of 13 Bacteroidetes strains significantly induced and improved larval settlement in SSW (sterile seawater), far above the rate of larvae in SSW or the addition with three non-Bacteroidetes strain representatives (Figure 1C). Among them, strains SP-4 and SW-2 reached the highest 96.67% settlement rate. As members of the γ -proteobacteria and α -proteobacteria, three non-Bacteroidetes species (*Marinobacter* sp. strain AL, *Brevundimonas vesicularis* strain AP, *Aliivibrio cyclitrophicus* strain VI) were found abundant in surrounding seawater or associated with sponge in our previous study (Wu et al., 2018). They had, however, negative effect on sponge larval settlement process. After about two weeks, sponge metamorphosis was initiated; the oscula formed, indicative of healthy growth (Figure S1). To preliminarily clarify how Bacteroidetes species act on sponge larvae to bring about settlement and metamorphosis, the cellular morphology of 13 strains was observed by using scanning electron microscopy (SEM) (Figure 1B). The analysis showed that almost a half of the bacterial species (6/13) produced extracellular vesicles (EVs) (Figure S2). Two strains formed dense biofilms, and others without any special phenotypic characteristics were possibly linked to larval settlement. To determine the effect of the biofilm or EVs or possible chemical cues on larval settlement, several Bacteroidetes representatives that had stable ability to promote larval settlement were selected for further study.

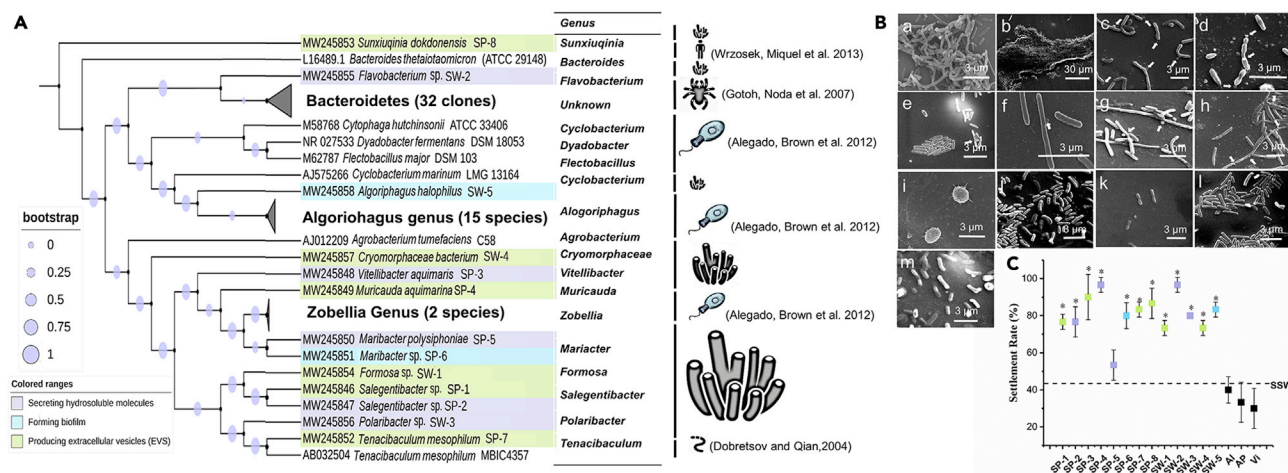


Figure 1. Identification of Bacteroidetes species derived from marine sponge and their effect on host larval development

(A) Phylogenetic tree of the cultivable Bacteroidetes species correlated with partial animal development. The phylogenetic tree was reconstructed by neighbor-joining method of the 16S rRNA gene sequences from Bacteroidetes bacteria including 13 species in this study and other species reported to function in animal developmental process which were downloaded from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Numbers refer to bootstrap values for each node out of a total of 1000 replicate samplings. All 13 Bacteroidetes species tested in *Tedania* sp. larval settlement assessment were shaded in different colors. Purple shade represented Bacteroidetes species secreting hydrosoluble molecules. Blue shade represented Bacteroidetes forming biofilm. Green shade represented Bacteroidetes producing extracellular vesicles.

(B) Scanning electron micrographs of 13 Bacteroidetes species. (a) SP-6 and (b) SW-5 showed that Bacteroidetes strains formed dense biofilms; (c-h) (SP-1; SW-1; SP-4; SP-7; SW-4; SP-5) indicated that Bacteroidetes species produced EVs labeled by white arrows; (i-m) (SP-2; SW-2; SP-3; SP-8; SW-3) showed Bacteroidetes strains without observable characteristics.

(C) The promoting rate of 13 Bacteroidetes species on *Tedania* sp. larval settlement compared to blank control SSW (sterile seawater). $n = 6$. Data are represented as mean \pm standard error of the mean (SEM). Asterisks indicate statistically significant differences (one-way analysis of variance [ANOVA], $*p < 0.05$). AL, *Marinobacter* sp. strain AL. AP, *Brevundimonas vesicularis* strain AP. VI, *Aliivibrio cyclitrophicus* strain VI. See also [Figures S1](#) and [S3](#) and [Tables S1](#) and [S2](#).

The effect of bacterial biofilms on the settlement rate of marine sponge larvae

The SEM images and biofilm formation results illustrated that only two Bacteroidetes strains (SP-6 and SW-5) had a strong capacity to form biofilms ([Figure 1B](#), [Table S2](#)). The monospecific biofilms of SP-6 and SW-5 were growing continuously and could clearly promote larval settlement, up to 63.33% higher than larvae in SSW (14.14%) ([Figure 2B](#)). After three days, the biofilms fragmented, and the boundary lines became vague, resulting in the biofilm structure becoming distorted ([Figure 2A](#)). The larval settlement rates of both monospecific biofilms incrementally declined to 16.67% after three days without supplementing fresh bacterial cells. In this study, a bacterial-forming biofilm from NSW (natural seawater) was made to assess the settlement-inducing activity compared with monospecific biofilms. The results showed that the NSW-forming biofilm had not yet reached the densest level by the third day; thus, the larval settlement rate was lower than that with monospecific biofilms ([Figures 2A](#) and [2B](#)). As the biofilm age increased with time, the settlement activity of larvae in the NSW biofilm increased, reaching 82.22% on day 15 ([Figure 2B](#)). The larvae exposed to clean surfaces in SSW as the blank control had a settlement rate of \sim 14.14% ([Figure 2B](#)).

Bacterial extracts promote the settlement rate of marine sponge larvae

Because no apparent characteristics of strains SW-2, SW-3, SP-2, SP-4, and SP-5 involving host settlement were observed under SEM, different components produced by strains were examined subsequently for their effect on larval settlement. SP-2 was selected as one representative for the stability in improving the larval settlement. Both aqueous and organic phases were extracted by ethyl acetate from cell pellets and supernatants of SP-2 cultures. Positive effects on larval settlement were found in aqueous phases using both bacterial supernatants and cell pellets, with the settlement rates reaching 59.17% and 44.17%, respectively ([Figure 3](#)). Although the specific compounds were not identified, the results verified that these Bacteroidetes species, as for strain SP-2, could secrete chemical cues favoring sponge larval development.

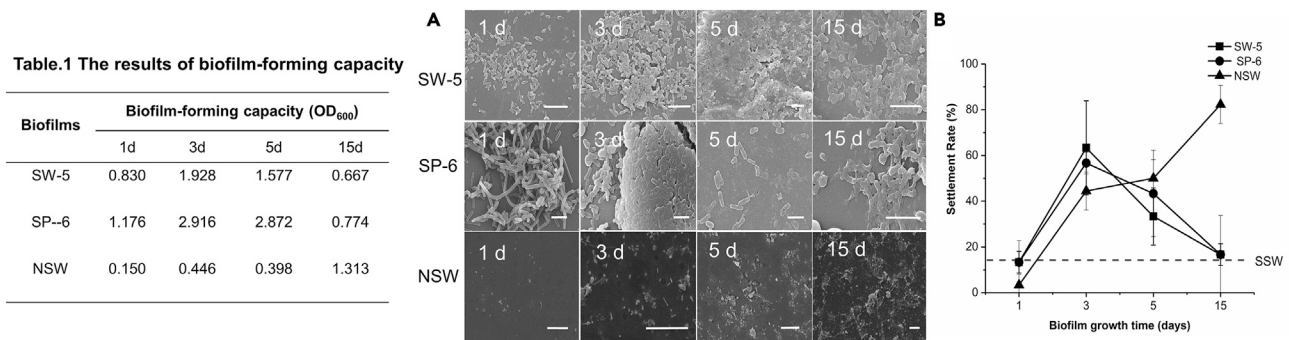


Figure 2. The effect of biofilms formed by *Bacteroidetes* species on sponge larval settlement
(A) Scanning electron micrographs of SW-5, SP-6, and NSW biofilms with time extension. Scale bar represents 3 μm (SW-5, SP-6), 30 μm (NSW).
(B) The settlement rate of *Tedania* sp. larvae influenced by strain SW-5, SP-6, and NSW biofilms. n = 6. Data are represented as mean ± SEM.
See also Figure S3.

Bacteria-derived EVs promote the settlement of marine sponge larvae

Based on the images of transmission electron microscopy (TEM), six strains were cultured in a marine broth to extract spherical outer-membrane vesicles. The method was confirmed to be effective in separating EVs from bacteria culture under TEM (Figure S2). These extracellular pellets were quantified according to the concentration of EV proteins and were subjected to bioassays for settlement-inducing capacity. Except strain SP-8 containing EVs with low concentration, EVs from other 5 *Bacteroidetes* strains (SW-1, SW-4, SP-1, SP-4, and SP-7) had the capacity to induce and improve the settlement of larvae, up to the highest rate of 86.67% (Figure 4A). Although to promote settlement activity, sponge larvae presented different requirements for the quantity of EVs, all EVs from each *Bacteroidetes* genus at the medium concentration reached the highest settlement rates. To understand the correlation between bacterial EVs and larval settlement, SP-7 was chosen for further study due to the stability of settlement promotion under different amounts of EVs. Nanoparticle tracking analysis revealed that the diameter of EVs was about 50–200 nm, and the EVs had a clear membrane structure (Figures 4B and 4C). Repeated results verified that only SP-7 cells detached from EVs did not induce larval settlement, while both EVs and bacterial fermentation had similar effects (Figure 4E). An attempt was made to assess the settlement-inducing activity of proteins produced by SP-7, but the results were negative (Figure 4D).

In the initial stage, EVs first attached to the surface of sponge larvae, as clearly shown under SEM and TEM (Figures 5A and 5B). To investigate whether and how EVs were internalized by larvae, fluorescent labeled SP-7-producing EVs were added to SSW containing swimming larvae. It was confirmed by using confocal microscopy that EVs were gradually transported from the surface of the larvae into the inner layers during incubation (Figure 5B). First, EVs labeled by a green fluorescent dye were distributed at the larval surface labeled with a blue fluorescent dye within 15 min, then most EVs migrated into the inner larval layers, with EVs continuing to be internalized at 30 min, and finally EVs became concentrated in the middle of the larvae after 10 hr. After larvae settled on the substrate, the distribution of EVs changed. During larval metamorphosis, the EVs spread to the edges of the larvae (Figure 5C). The visual process of EVs migration provided the evidence for the role of EVs in larval settlement.

DISCUSSION

The phylum *Bacteroidetes* constitutes a large portion of marine bacterioplankton that degrades particles and polymers (Amaral-Zettler et al., 2010). Members of the phylum *Bacteroidetes* are also abundant in the ocean and globally distribute in a variety of marine environments such as coastal, offshore, sediment, and hydrothermal vents (Fernández-Gómez et al., 2013; Pita et al., 2018) that enable them to come into close contact with marine creatures. Phylum *Bacteroidetes* has a relatively high distribution associated with animals, from lower unicellular animals to mammals. Actually, nearly all animals have stable interactions with bacteria, but it is difficult to investigate how these relationships shape host function, particularly developmental processes, due to the complex bacterial composition and the consistently changing environment. Our previous results showed that some *Bacteroidetes* members associated with sponge larvae sharply increased from swimming to settlement stages, suggesting an important role of bacterial species on

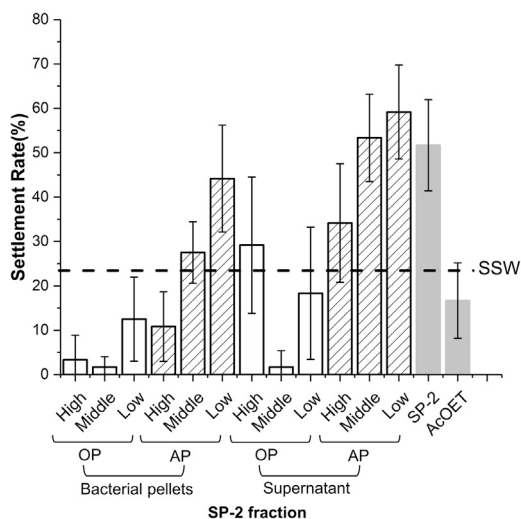


Figure 3. The impact of extracted fractions from SP-2 on sponge *Tedania* sp. larval settlement

AP, aqueous phase; OP, organic phase. Each phase was extracted from SP-2 bacterial pellets or supernatant and diluted to a series of concentrations (Table S1). $n = 3-6$. Data are represented as mean \pm SEM. SP-2 was a bacterial culture treated as a positive control. AcOEt was used as a solvent blank control. See also Figure S3 and Table S1.

host development (Wu et al., 2018). In this study, 13 species (12 genera) within three Bacteroidetes classes dominant in marine environments showed significant promotion of *Tedania* sp. larval settlement and metamorphosis. Despite great diversity at every level of gene sequence resolution, members of the phylum Bacteroidetes share certain attributes that reflect their shared ancestry (Figure 1A) (Johnson et al., 2017). Therefore, the small representation limited to the culturable bacteria partially demonstrated the mutualism between Bacteroidetes species and sponge larvae.

Many benthic invertebrates respond to microbial biofilms that serve as important cues to induce faster and more successful settlement and metamorphosis of larvae (Freckelton et al., 2017). The accumulating evidence has appeared in sponges (Webster et al., 2004), cnidarians (Leitz and Wagner, 1993; Morse and Morse, 1991), bryozoans (Hitoshi et al., 1987; Roberts et al., 1991), mollusks (Avendao-Herrera et al., 2003; Dobretsov and Sergey, 1999; Tamburri et al., 2008), annelids (Qian, 1999), echinoderms (Huggett et al., 2006), crustaceans (Bourne et al., 2006), and urochordates (Woznica and King, 2018). In this study, two strains could form dense biofilms within three days and significantly promote sponge larval settlement, though strongly influenced by biofilm age and type. The density of micro-biofilm depends on the nutritional conditions and interaction between bacterial strains. NSW is a complex mixture compared to SSW and includes abiotic and biotic factors. In the interaction patterns of larval settlement and metamorphosis responding to biofilms, whether in complex microbial biofilm or in monospecies biofilms, Bacteroidetes species was an important component (Dang and Lovell, 2016; Dobretsov and Qian, 2004; Webster et al., 2004). However, the number of larval types recorded to settle and metamorphose in response to bacterial films is now so great as to suggest a nearly universal mechanism for both settlement induction and larval response (Freckelton et al., 2017), yet we know very little about either of these factors.

Chemical compounds mediating ecological interactions including larval settlement are prevalent in marine environments (Zimmer and Butman, 2000). Those natural chemical cues could emanate from a wide variety of sources such as multi-species biofilms (Hung et al., 2009), host algae (Swanson et al., 2006), conspecifics (Matsumura et al., 1998), and prey species (Hadfield and Pennington, 1990). However, the chemistry approach has contributed to this area with relatively little information concerning microbial products. Tetrahydrofuran, a small and non-polar bacterial metabolite, is linked to partial or complete metamorphosis for some corals (Sneed et al., 2014). Bacterial lipopolysaccharide produced by *Cellulophaga lytica* is the inductive molecule that induces the settlement and metamorphosis of *Hydroides elegans* larvae (Freckelton et al., 2019). *Pseudoalteromonas luteoviolacea* has been subjected to the most detailed genetic and mechanistic studies that produce arrays of phage-tail-like structures triggering metamorphosis of *H. elegans* (Shikuma et al., 2014). Bacterial nucleobases produced by *Vibrio owensii* MS-9 play an important role in larval settlement and metamorphosis of marine invertebrates (He et al., 2019). Symbiotic bacteria larvae of *Amphimedon queenslandica* could produce the essential amino acid arginine, which the larvae require to synthesize nitric oxide—an essential signaling molecule for larval settlement and metamorphosis (Hao and Hewitt, 2020). In this study, the aqueous phase from Bacteroidetes bacterial cells or

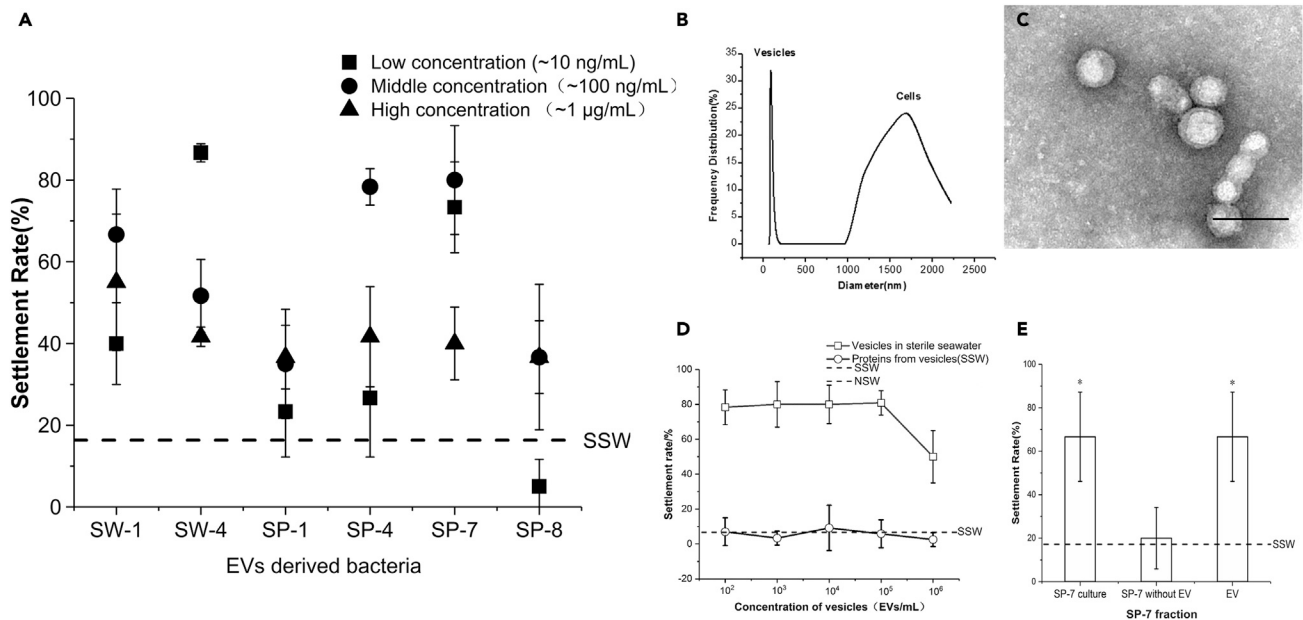


Figure 4. The effect of Bacteroidetes species-derived EVs on sponge *Tedania sp.* larval settlement

(A) The settlement rate of sponge larvae treated with EVs in different quantities produced by six Bacteroidetes species. $n = 3-6$. Data are represented as mean \pm SEM.

(B) Particle size distribution of purified EVs and bacterial cells from the SP-7 culture determined by nanoparticle size analysis.

(C) Purified SP-7-derived EVs observed by negative staining under TEM. Scale bar, 100 nm.

(D) The settlement rate of sponge larvae treated with strain SP-7 EVs with different conditions. E, the settlement rate of larvae influenced by SP-7 different fractions. $n = 3$. Data are represented as mean \pm SEM.

(E) The settlement rate of SP-7 fractions. $n = 3$. Data are represented as mean \pm SEM. See also Figure S3 and Table S1.

secretions indicated that the hydrosoluble molecules could be settlement-inducing chemical cues for *Tedania sp.* larvae. In an aqueous environment, communication between living organisms depends on solubility of the chemical compounds in water, regardless of complex structures and large molecular weights (Bhakuni and Rawat, 2005). However, the related comprehensive literature on marine benthic larvae settlement, particularly in marine sponge, was only reported sporadically. Even though no concrete substances were identified here, Bacteroidetes strains demonstrated a way of affecting sponge larval developmental biology by producing hydrosoluble chemical compounds.

Obviously, bacterial biofilms and compounds were effective cues for sponge larval settlement but are not absolute requirements. Here, the larvae of *Tedania sp.* typically settled in the absence of a microbial biofilm because bacterial outer membrane vesicles with sizes ranging from 50 to 200 nm in diameter could provide signal compounds. The bacterial EVs, as the bridge between cell-to-cell communication and the underlying environmental triggers, have been identified in a number of pathogenic bacteria and in higher animals (Aschtgen et al., 2016). Compared to the literature available (Reyes-Robles et al., 2018), there are few reports about marine host-microbe interactions in terms of symbiotic bacterial EVs. EVs produced by *V. fischeri* are powerful contributors to the development of squid light organs (Aschtgen et al., 2016). EVs from *Algoriphagus* contained signal lipids that induce rosette development in *S. rosetta* (Woznica et al., 2016). These vesicles with simple biological structures have been demonstrated to protect biomolecules from degradation, to be involved in long-distance transport to target receptor cells, and to aggregate effective concentrations to affect biological processes (Farcas and Inngjerdigen, 2020; Xue et al., 2021; Yang et al., 2018). It is very important, particularly in the ocean, to recognize that the fluidity and dilution capacity of seawater led to passive interactions between bacteria and hosts during the course of evolution, and the creation of the first cell required sequestration of valuable molecules from the hazardous external microenvironment. Signal cues were packed into EVs and further delivered with varying propensities to different recipient cells (Toyofuku et al., 2017). So far, it cannot yet quantify the cargo and specify the functional component. The results that EVs at the medium concentrations reached the best inductive

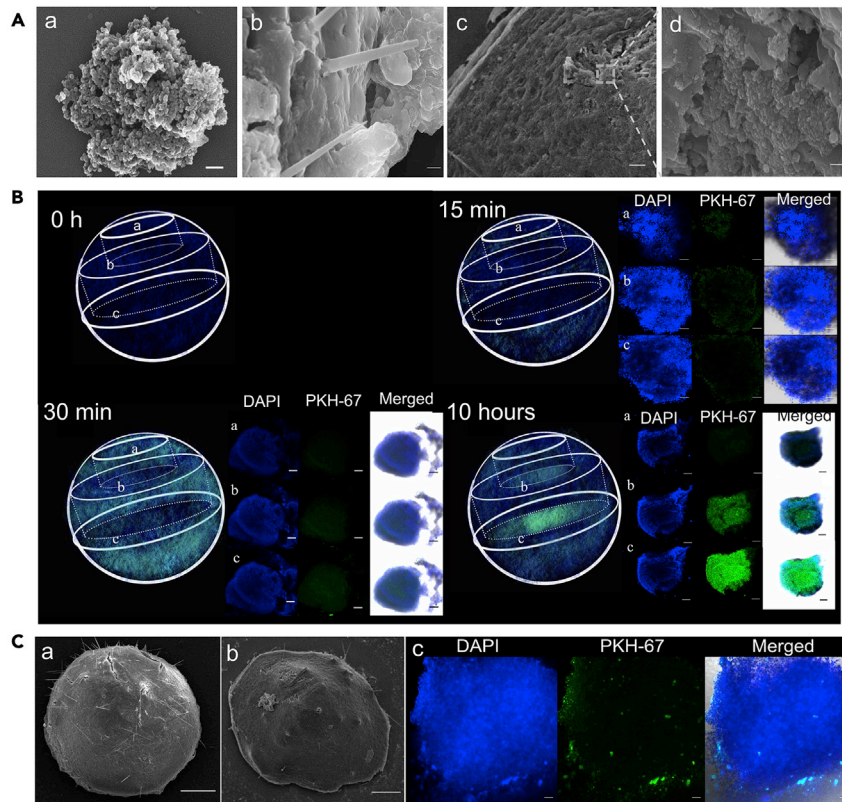


Figure 5. Distribution of EVs and their pathway into sponge larvae from swimming stage to settlement and metamorphosis of marine sponge

(A) Swimming larva consistently attached by EVs, including diagram (a), concentrated and purified EVs observed by SEM. Scale bar, 200 nm; (b), the surface of larva without any EVs observed by SEM. Scale bar, 2 μ m; (c), the out layer of the larva co-cultivated with EVs. Scale bar, 2 μ m; (d), the partial enlarged detail from (c). Scale bar, 200 nm.

(B) EVs' internalization by larva. Larva at 0 hr without labeled EVs was stained blue using DAPI. At 15 min, EVs attached to the outer layer of the larva. In the image, the white layer (a) and the outer ring of (b) and (c) layers were only slightly covered by green fluorescence because the centers of (b) and (c) layer were still blue. At 30 min, EVs consistently internalized, and all white (a), (b), and (c) sections were overlaid by EVs. After 10 hr, EVs gathered in the inner part of larva, with the strongest green fluorescence appearing in the (c) section. Scale bars, 50 μ m.

(C) The settlement and metamorphosis of marine sponge larva. (a) The image shows the settled larva with conical morphology under SEM. Scale bar, 100 μ m; (b) the larva in metamorphic process with a flat form by SEM. Scale bar, 100 μ m. (c) EVs distributed to the outer edge of metamorphic larva. Scale bars, 50 μ m. See also [Figure S3](#).

effect seemed to predict the complexity of EV content. It suggested that these membrane-forming vesicles could pack benefit or harmful metabolites to host development. Extracting EVs under different conditions, like bacterial growth curve, could be in favor of determining the specific compound in future work. Evidence given in this study further confirms that EVs successfully internalized and then concentrated in the inner sections of the larvae before metamorphosis. For sessile marine invertebrates such as sponges, the settlement of pelagic larvae is not only a critical process for population maintenance and biogeography but also the turning point in sponge life history and ontogeny ([Abdul Wahab et al., 2014](#)). Due to the restricted nutrition and swimming capability, seeking more opportunities to effectively settle on suitable substrates is crucial for sponge larvae ([Baird et al., 2003](#); [Mundy and Babcock, 1998](#)). In marine environments, animals co-evolved with bacteria ([Gruber-Vodicka et al., 2019](#)). They selectively accumulated epibiotic bacteria from the seas that could influence the biology of the host ([Bonhond et al., 2020](#); [Saha and Weinberger, 2019](#)). For the sake of survival, bacteria also evolved strategies favoring host metabolism and behavior because they were unable to insulate themselves from various external physical and chemical factors ([Dobretsov and Rittschof, 2020](#)). In this study, Bacteroidetes bacteria applied three strategies (biofilms, secreting compounds, and especially use of EVs) to significantly trigger and improve larval settlement, an important factor for being enriched in competent larval stages of sponges ([Wu et al., 2018](#)).

However, the influence of one typical bacterial species on animals is limited; the influence of a bacterial phylum on animals employing the same strategy from that used in primitive animals to the modern animals could be more profound. *Algoriphagus machipongonensis* regulated the development of choanoflagellates, the closest living relatives of animals, and choanoflagellate-like cells likely formed the basis for the evolution of animal epithelial cells (Woznica et al., 2016). *Cardinium* sp., the intracellular symbiotic bacteria, induces parthenogenesis and feminization of genetic males (Gotoh et al., 2007). Moreover, in the colonic epithelium of vertebrates, *B. thetaiotaomicron* affects the differentiation of goblet cells and mucin synthesis (Wrzosek et al., 2013). More evidence provided by many researchers illustrates that animals share an ancestor that contained obligate, vertically transmitted bacterial symbionts (Moran et al., 2005). Bacteroidetes, as one important host-associated bacterium, could have intimate relationships with most hosts among the whole of evolutionary history by influencing individual development and differentiation.

In conclusion, we provide evidence that (i) post-settlement stage-specific phylum Bacteroidetes promote sponge larval settlement; (ii) Bacteroidetes species evolved three strategies to achieve the settlement induction, by forming biofilms, by using chemical cues, and by EV delivery; and (iii) 50% of Bacteroidetes species in this study were shown to produce EVs involved in host developmental processes. Many factors are associated with bacteria-host interactions as well as signal delivery; the same process could be influenced by physical structure, signal cues, or other unknown mechanisms. From these initial observations, the understanding of the factors that drive the symbioses and co-evolution between bacterial phyla and animals has been broadened. The knowledge of a microbial group's role in animal origins may strengthen our perspective of the necessary functional and ecological dimensions of holobionts.

Limitations of the study

In this study, EVs of Bacteroidetes strain SP-7 were observed to internalize and migrate inside the sponge larvae, subsequently inducing their settlement. But the specific functional component interacted with target cell of sponge larvae had not been verified. Future research would figure out the effective signal molecule in EVs and deeply study the mechanism among this process.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.102662>.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.L. and J.Z.; methodology, M.L. and J.Z.; investigation, M.L., K.W., and C.J.; writing-original draft, M.L.; writing-review & editing, J.Z.; funding acquisition, J.Z.; resources, T.L., S.Y., and H.O.; supervision, M.L. and J.Z.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Salegentibacter</i> sp. strain SP-1	This study	MW24846
<i>Salegentibacter</i> sp. strain SP-2	This study	MW24847
<i>Vitellibacter aquimaris</i> strain SP-3	This study	MW24848
<i>Muricauda aquimarina</i> strain SP-4	This study	MW24849
<i>Maribacter polysiphoniae</i> strain SP-5	This study	MW24850
<i>Maribacter</i> sp. strain SP-6	This study	MW24851
<i>Tenacibaculum mesophilum</i> strain SP-7	This study	MW24852
<i>Sunxiuqinia dokdonensis</i> strain SP-8	This study	MW24853
<i>Formosa</i> sp. strain SW-1	This study	MW24854
<i>Flavobacterium</i> sp. strain SW-2	This study	MW24855
<i>Polaribacter</i> sp. strain SW-3	This study	MW24856
<i>Cryomorpaceae bacterium</i> strain SW-4	This study	MW24857
<i>Algoriphagus halophilus</i> strain SW-5	This study	MW24858
<i>Brevundimonas vesicularis</i> strain AP	This study	MW24859
<i>Marinobacter</i> sp. strain AL	This study	MW24860
<i>Vibrio cyclitrophicus</i> strain VI	This study	MW24861
Chemicals, peptides, and recombinant proteins		
Marine Broth 2216 Agar	Difco	212185
Chloramphenicol	Macklin	C804169-100g CAS#: 56-75-7
Tetracycline HCl	Solarbio	T8180-10g CAS#: 64-75-5
streptomycin Sulfate	Solarbio	S8290 CAS#: 3810-74-0
Ethyl acetate	Xilong	CAS#: 141-78-6
Bovine serum albumin	Takara	2320 CAS#:17879-45-7
PKH67 Green Fluorescent Cell Linker Mini Kit	Sigma-Aldrich	Cat# MINI67
Triton X-100	Sigma	Cat# T8787
4',6-diamidino-2-phenylindole (DAPI)	Life Technologies	62248 CAS#: 28718-90-3
Critical commercial assays		
Bacterial Protein Extraction Kit	Sangon Biotech	C600596-0001
Bradford Protein Quantification Kit	Vazyme	E111-01
Oligonucleotides		
Primer: 27 former: 5'-AGAGTTTGATCCTGGCTCAG-3'	Sangon	(Lane, 1991)
Primer: 1492 reverse: 5'-TACGGYTACCTTGTTACGACTT-3'	Sangon	(Lane, 1991)
Deposited data		
Data deposited at Mendeley Data	This study	https://doi.org/10.17632/y3kcw2rmhk.1
16S rRNA sequencing data from all samples	This study; Genbank	accession number from MW24846 to MW24861
Biological samples		
<i>Tedania</i> sp. larvae	This study	N/A
Software and algorithms		
MEGA6	MEGA software	https://www.megasoftware.net/
OriginPro8	Originlab	https://www.originlab.com/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zen 2011	Zeiss	https://www.zeiss.com.cn/microscopy/products/microscope-software/zen-lite.html
SPASS 22.0	IBM	https://www.ibm.com/cn-zh/analytics/spss-statistics-software
Other		
0.22 μm pore-size PVDF membrane filters	Millipore	ISEQ10100
0.45 μm pore-size PVDF membrane filters	Millipore	IPVH00010
100 kDa filter membrane	Millipore	UFC810096
confocal 35-mm Petri dishes	Solarbio	YA0572

RESOURCE AVAILABILITY

Lead contact

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Materials availability

This study did not generate any new reagents.

Data and code availability

All data supporting findings of this study are provided within the manuscript and its [supplemental information](#) section. Whole 16S rRNA sequencing data of 13 Bacteroidetes strains has been deposited on the GenBank with the accession number from MW24846 to MW24861. Original data of this paper has been deposited at Mendenley data (<https://dx.doi.org/10.17632/y3kcw2rmhk.1>).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sponge larvae

Tedania sp. was farmed by ropes under the seawater about 0.5–2 meters in a floating sea raft, in 2018–2020, in Dongshan Bay, Zhangzhou City, Fujian Province, China (geographic coordinates 23.80°N and 117.59°E) (Figure S3). From May to August, most of the adults released larvae naturally in the early morning beginning at approximately 6:00 am, with the most active release during 6:00 am–9:00 am. To collect the released larvae, a reproductive adult was netted with a mesh screen (150 μm) at 6:00 am and then removed three hours later. The larvae were 400–500 μm in size, and 30–50 larvae were trapped as one parallel sample. All of the samples in this study were transported to the lab and immediately treated in settlement assays in 2019. The seawater was sampled at a depth of ~50 cm from the top water layer, similar to sponge aquaculture.

METHOD DETAILS

Strains isolation and identification

All bacterial strains in this study were isolated from marine sponges and surrounding seawater by standard dilution plating techniques on marine agar 2216E plates (Difco, Detroit, MI, USA) at 28°C. Bacterial isolates were selected, purified, and identified. The 16S rRNA gene was amplified from pure colony using bacterial universal primers (27F and 1492R) (Lane, 1991). The amplificative reaction conditions were 95°C for 5 min; 30 cycles (90°C for 5 min; 55°C for 30 s; 72°C for 90 s) and 72°C for 10 min (Wu et al., 2018). Sequencing of 16S rRNA genes was performed by Shanghai Life Technology Co, PR China. All 16S rRNA gene sequences (~1500 bp) belonging to phylum Bacteroidetes (accession number from MW24846 to MW24858) and three non-Bacteroidetes species (accession number from MW24859 to MW24861) were chosen for further analysis and were submitted to GenBank.

Larval settlement assay

Total 13 species of Bacteroidetes and three non-Bacteroidetes species (*Marinobacter* sp. strain AL, *Brevundimonas vesicularis* strain AP, *Aliivibrio cyclitrophicus* strain VI) were tested to assess the impact on larval

settlement. These non-Bacteroidetes species were selected because of the relatively richness in surrounding seawater or sponge in our previous analysis (Wu et al., 2018) and in culture-dependent bacteria. Natural seawater (NSW) was treated to be sterile seawater (SSW) by using a mixture of antibiotics: chloramphenicol (1.2 g/L), tetracycline (1.2 g/L), and streptomycin (0.12 g/L) for four days. All bacteria were quantified up to 10^3 cells/mL and afterward added into SSW (Inaba and Mizuno, 2009) and NSW with 30–50 larvae in each experimental group. Six parallel plates were set for each trial group. After 8–12 hr, the numbers of settled larvae were counted to evaluate the larval settlement. Those larvae that attached firmly to the substrates and were not easily dislodged by Pasteur pipettes (2 mL) were regarded as ‘effectively settled’.

Bacterial biofilm formation

Biofilms of all Bacteroidetes strains were tested by crystal violet assay and the absorbance at 600 nm were measured using a spectrophotometer (Shukla and Rao, 2017) (Table S2). SP-6 and SW-5 were found to have strong biofilm forming ability under SEM observation. Monospecific biofilms were generated from SP-6 and SW-5. Bacterial strains were inoculated into 2216E broth and cultured at 28°C for 24 hr in a shaking incubator (170 rpm). Then, 100 μ L aliquots of each bacterial culture were transferred into a fresh tube for dilution prior to counting. About 8 mL fermentation broth containing 10^3 cells/ml were added into each well of 6-well plates in which single circular silicon wafers (10 mm diameter) had been placed previously for biofilm detection by SEM. Additionally, NSW biofilms were formed as positive controls by adding NSW in 6-well plates and SSW as negative controls. Each trial group was treated in six parallel replicates. All plates were incubated at 28°C for 1, 3, 5, and 15 days. At the designated time points, the supernatant was discarded and washed twice to remove unattached bacteria. Afterward, about 30–50 sponge larvae were added for the settlement assay as described above.

Chemical compounds extraction

Strain SP-2 belonging to phylum Bacteroidetes was selected as one representative with no particular cell structure. The cell pellets and supernatants from 100 mL of SP-2 fermentation broth were separated by centrifuging at 4000 \times g for 15 min at room temperature. The possible bioactive components from cell pellets or supernatants were isolated in equal volumes of ethyl acetate. After 2 hr extraction, the organic and aqueous phases were separated. The solvent was removed by rotary evaporation at 35°C, 0.095 MPa (AnkeYq, Shanghai), and the possible bioactive compounds were lyophilized and extracted in the lyophilizer at –50°C, 20Pa (Biocool, Beijing). Finally, four fractions from SP-2 cell pellets and supernatants diluted by ddH₂O and ethyl acetate respectively to different levels were tested for larval settlement assays as described above (Table S1).

Bacteria-derived EVs isolation

The extracellular vesicles (EVs) were isolated from bacterial culture supernatants based on a modified procedure (Kulp and Kuehn, 2010). All strains were grown in 2216E media at 28°C to middle exponential phase. Most bacterial cells were removed by centrifugation at 4,500 \times g for 15 min. The residual bacterial pellets were successively filtered through 0.45 μ m and 0.22 μ m pore-size PVDF membrane filters (Millipore Co. Ltd). The cell-free supernatant was then concentrated to a small volume with a 100 kDa filter membrane (Millipore). EVs were separated from other extracellular products by ultracentrifugation at 100,000 \times g for 2 hr at 4°C (41 Ti rotor, Beckman Coulter). The resulting pellets were washed and resuspended in PBS (Solarbio). The number of EVs was roughly estimated by the concentration of EV protein (Klimentová and Stulík, 2015). EV proteins were extracted using a Bacterial Protein Extraction Kit (Sangon Biotech) and quantified by using the Bradford Protein Quantification Kit (Vazyme) following the manufacturer’s protocol. Both EVs and EVs-derived proteins were diluted to various concentrations that were artificially defined as high (~1 μ g/mL), middle (~100 ng/mL), and low (~10 ng/mL) levels for the sponge larval settlement assay (Table S1).

Bacterial EVs internalization analysis

Bacteroidetes strain SP-7 was selected for further study based on the stability of larval settlement activity. SP-7-derived EVs were labeled with the PKH67 Green Fluorescent (Cell Linker Kit, Sigma-Aldrich) with minor modifications by decreasing the concentration of the dye. To bind excess dye, 2 mL 0.5% BSA/PBS was added to the mixture, and the labeled EVs were washed twice and resuspended in PBS to remove excess PKH-67 by ultracentrifugation as mentioned above. Afterward, the labeled EVs were incubated with larvae in NSW in confocal 35-mm Petri dishes. Larvae samples were collected after 15 min, 30 min, 1 hr, and 10 hr,

washed three times with PBS, and finally fixed with 4% formaldehyde solution at room temperature for 10 min. Following three additional washes in PBS, the larvae were treated with 1% Triton X-100 for 10 min to permeabilize the larval cells. Subsequently, the larval nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). Then, bacterial EVs internalization at different times was visualized and analyzed under a confocal laser scanning microscope (LSM780NLO; Carl Zeiss, Oberkochen, Germany).

QUANTIFICATION AND STATISTICAL ANALYSIS

Settlement rate of sponge larvae was calculated with the following formula:

$$\text{Settlement rate} = \frac{\text{Number of settled larvae}}{\text{Number of all larvae}} \times 100\%$$

Data were presented as means \pm SEM, $n = 3-6$, and n represented the 30-50 sponge larvae under the same experimental treatment. The data were analyzed for statistical differences with SPASS, using ANOVA (one-way analysis of variance). A probability value less than 0.05 ($p < 0.05$) indicated significant difference between experimental groups and control groups.