Two distinct bacterial biofilm components trigger metamorphosis in the colonial hydrozoan *Hydractinia echinata*

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Abstract

Bacterial-induced metamorphosis of larvae is a widespread cross-kingdom communication phenomenon within the marine environment and critical for the persistence of many invertebrate populations. However, the chemical structures of the majority of inducing bacterial signals and the underlying cellular mechanisms remain enigmatic. *Hydractinia echinata* larvae transform upon detection of bacterial biofilm components into the colonial adult stage. Despite serving as cell biological model system for decades, the inducing bacterial signals remained undiscovered. Using a chemical-ecology driven analysis, we herein identified that specific bacterial (lyso)phospholipids and polysaccharides, naturally present in bacterial biofilms, elicit metamorphosis in *Hydractinia* larvae. While (lyso)phospholipids (e.g. 16:0LPG/18:1LPE, 16:0LPA/18:1LPE) as single compounds or in combinations induced up to 50% of all larvae to transform within 48 h, two structurally distinct polysaccharides, the newly identified Rha-Man polysaccharide from *Pseudoalteromonas* sp. P1-9 and curdlan from *Alcaligenes faecalis* caused up to 75% of all larvae to transform within 24 h. We also found combinations of (lyso)phospholipids and curdlan induced the transformation in almost all larvae within 24 h, thereby exceeding the morphogenic activity observed for single compounds and axenic bacterial biofilms. By using fluorescence-labeled bacterial phospholipids, we demonstrated their incorporation into the larval membranes, where interactions with internal signaling cascades could occur. Our results demonstrate that multiple and structurally distinct bacterial-derived metabolites converge to induce high transformation rates of *Hydractinia* larvae, which might ensure optimal habitat selection despite the general widespread occurrence of both compound classes.

Significance Statement

Bacterial biofilms profoundly influence the recruitment and settlement of marine invertebrates, critical steps for diverse marine processes such as coral reef formation, marine fisheries and the fouling of submerged surfaces. Yet, the complex composition of biofilms often makes it challenging to characterize the individual signals and regulatory mechanisms. Developing tractable model systems to characterize these ancient co-evolved interactions is the key to understand fundamental processes in evolutionary biology. Here, we characterized for the first time two types of bacterial signaling molecules that induce the morphogenic transition and analyzed their abundance and combinatorial activity. This study highlights the crucial role of the converging activity of multiple bacterial signals in development-related cross-kingdom signaling.
Main Text

Introduction
The radical transformation (metamorphosis) of planula larvae into the adult stage is a critical step in the life cycle of many marine species as it confers the propagation and persistence of the population in the marine ecosystem. For more than 80 years it has been recognized that chemical signals present within marine bacterial biofilms induce or even prevent settlement and metamorphosis in benthic marine larvae, but their identification remains still a challenging task due to low production levels and unestablished model systems. Hence, until today only very few key bacterial signals have been structurally characterized. A prime example represents the bacterial product thallusin isolated from Zobellia uliginosa, which induces metamorphosis in the alga Monostroma oxyspermum. Several members of the Polychaeta class and Cnidaria phylum have also served as model systems for bacterial-induced metamorphosis over decades. In several studies, it was found that bromopyrroles produced by Pseudoalteromonas induce larvae of several coral species to undergo metamorphosis; however induced larvae failed to attach to surfaces when stimulated by bromopyrroles alone indicating that other, yet unidentified, chemical cues might be important for the morphogenic process. Recent biochemical investigations of the bacteria-induced metamorphosis of the marine polychaete Hydroides elegans resulted in the identification of a phage tail-like contractile injection systems (tailocins) in Pseudoalteromonas species that induce settlement and metamorphosis by releasing an effector protein Mif1, which stimulates the P38 and MAPK signaling pathways. However, bacteria not capable of producing these proteinaceous injection systems were also found to induce the transformation releasing additional, yet structurally not defined morphogens. In the 1970s, Leitz and Wagner reported that a lipid-like molecule of Pseudoalteromonas espejiana (original name: Alteromonas espejiana) induces larvae transformation in Hydractinia echinata, an early branching metazoan lineage dating back more than 500 million years. But despite intensive studies, the bacterial signals causing Hydractinia larvae to metamorphose have remained elusive. Instead, metamorphosis of Hydractinia was artificially induced using high salt concentrations (CsCl) allowing seminal studies on migratory stem cells, alloreognition (self-recognition), the canonical Wnt-signalling system, and the development of muscles and nervous systems. However, it was noted from early on that artificial induction caused phenotypical and developmental differences in Hydractinia development compared to bacterial induction.

The long-standing unsolved question about the structures of bacterial signals and the apparent morphological differences in larvae development between artificial and natural induction attracted our interest. Thus, we set out to solve the structures of the bacterial signals that induce metamorphosis in Hydractinia, which would allow us to shed light on the biochemistry underlying this ancient prokaryote-eukaryote signaling mechanisms.
Results

Bioassay-guided identification of bacterial signals

To investigate which co-occurring bacteria induce metamorphosis in *H. echinata* (hereafter *Hydractinia*) we isolated morphological distinct co-occurring bacterial species from the surface of a healthy and freshly collected *Hydractinia* colony. For a mono-species biofilm-based metamorphosis assay, we selected 29 representative bacterial isolates, including seven genome-sequenced *Hydractinia*-associated strains, one coral associated strain *Pseudoalteromonas* sp. PS5, and eight bacterial type strains obtained from culture collections. Similar to previous observations, we observed the inconsistent timing of metamorphosis using biofilms compared to the artificial control (> 6 mM CsCl final concentration), presumably due the inhomogeneous nature of biofilms and spatial concentrations differences of the yet unidentified inducing signals in bacterial biofilms. To enable a comparative analysis of the inducing activity, we adopted the established stage-chart of morphological appearances by Leitz and coworkers to describe and calculate biofilm-induced rates of transformation (Figure S1). In short, the percentage of larvae settlement includes only counts of larvae in morphological stages 9-11, and the percentage of complete metamorphosis includes only counts of larvae in morphological stages 12-14. Based on these assessments, transformation rates are given as averaged percentage ranges in 10% step ranges, which are calculated from replicates of experiments performed with different larvae batches (different spawning events to ensure reproducibility of results).

As depicted in Figure 1, our bioassays resulted in the identification of five *Hydractinia*-associated bacterial strains that caused up to 60% of all larvae to undergo complete transformation to the primary polyp. Most notably, three strains (P1-9, P6-7, P1-29) rapidly induced the transformation reaching the morphological stages 13-14 within 24 h. Additional five strains were found to induce the transformation to stages 9-11 within the first 24 h, but subsequent development into the primary polyp was only observed within 48 h. In addition, we observed that up to eleven strains induced the transformation of larvae to morphogenic stage 9-11. However, neither of those strains induced the full transformation to the primary polyp leading eventually to the death of the transforming organism. In addition, six strains were found to be non-inducing and four strains caused the death of up to 100% of all larvae within 24 h. The results of this study suggested firstly that more than one structurally distinct bacterial signal might induce the transition and secondly that the mode of action might differ depending on the structure of secreted molecule. Amongst all tested strains, *Pseudoalteromonas* sp. P1-9 (referred from now on as P1-9) was found to induce the most robust morphogenic response and thus was selected for further chemical analysis (Figure 2).
Figure 1. Assessment of morphogenic activity of selected bacterial strains. A) Left: phylogenetic tree based on 16S rRNA gene sequences of 29 tested bacterial strains. Best DNA model was generated and the robustness of interfered tree topologies was evaluated after 1000 bootstraps (> 50% are shown). Right: Heatmap depicts transformation percentage (stage 9-11 and 12-14) of *H. echinata* larvae counted after 24 and 48 h (negative control: ASW; positive control: CsCl). Transformation rates are given as averaged percentage ranges in 10% step ranges, which are calculated from replicates of experiments performed with different larvae batches. B) *Hydractinia echinata* colonizing the shell inhabited by a hermit crab (*Pagurus* sp.). C-E) Microscopic image of C) planula larva; D) initiation of morphogenic transformation by disc formation (morphological stage 9-10); E) formation of primary polyp (morphological stage 14).

To test if the morphogenic cue of P1-9 is a secreted and/or a diffusible small metabolite, we first tested solid-phase extracts (C18 cartridges) derived from liquid and plate cultures of P1-9. However, none of the tested solid-phase extracts showed morphogenic activities compared to living bacterial biofilms (positive control) (entry 3, Figure 2). We then tested if the signal is a
secreted high-molecular weight (HMW) biomolecule (e.g. protein, exopolysaccharides (EPS))
and/or part of the bacterial membranes.

Size exclusion based separation of culture supernatants and cell membrane fragments resulted in
the isolation of a HMW fraction (> 30 kDa) that showed remarkably high morphogenic activity in a
dose-response manner (entry 5). In contrast, low-molecular weight fractions (< 5 kDa) showed
only moderate to very low morphogenic activities (entry 6).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>1</td>
<td>Sea water control (ASW)</td>
<td>Negative control</td>
</tr>
<tr>
<td>2</td>
<td>CsCl (6 mM)</td>
<td>++ Positive control</td>
</tr>
<tr>
<td>3</td>
<td>Bacterial cells from plate and liquid culture</td>
<td>++ Part of cells or extracellular matrix</td>
</tr>
<tr>
<td>4</td>
<td>Solid phase (C-18) extracts of culture supernatants</td>
<td>- No secreted small molecule</td>
</tr>
<tr>
<td>5</td>
<td>Size exclusion filtration of culture supernatants (&gt; 30 DA)</td>
<td>++ Part of membrane or extracellular matrix</td>
</tr>
<tr>
<td>6</td>
<td>Size exclusion filtration of culture supernatants (&lt; 5 kDa)</td>
<td>+ Non secreted small molecule</td>
</tr>
<tr>
<td>7</td>
<td>Outer membrane vesicles and/or membrane layers</td>
<td>++ Bound to cells or extracellular matrix</td>
</tr>
<tr>
<td>8</td>
<td>EPS Isolation</td>
<td>++ Extracellular polysaccharides</td>
</tr>
<tr>
<td>9</td>
<td>DNase I</td>
<td>++ No DNA</td>
</tr>
<tr>
<td>10</td>
<td>RNase A</td>
<td>++ No RNA</td>
</tr>
<tr>
<td>11</td>
<td>Proteinase K</td>
<td>++ No proteins</td>
</tr>
<tr>
<td>12</td>
<td>Heating at 90°C</td>
<td>++ Heat resistant</td>
</tr>
<tr>
<td>13</td>
<td>6 M NaOH, 30°C, 12 h</td>
<td>++ Base stable - soluble</td>
</tr>
<tr>
<td>14</td>
<td>6 M HCl, 30°C, 12 h</td>
<td>++ Partial acid stable</td>
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<tr>
<td>15</td>
<td>6 M HCl, 60 − 90°C, 12 h</td>
<td>− Hydrolysis</td>
</tr>
<tr>
<td>16</td>
<td>Extraction of cell membrane</td>
<td>+ Water soluble</td>
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<td>17</td>
<td>CHCl₃ or cyclohexane</td>
<td>+ small molecule − part of cell membrane</td>
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<tr>
<td>18</td>
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<td>++ Polar lipids</td>
</tr>
<tr>
<td>19</td>
<td>MeOH/CHCl₃</td>
<td>++ Polar lipids</td>
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</table>

**Figure 2.** Morphogenesis assay of biosamples derived from P1-9. Transformation rates to
morphological stages 12-14 were calculated from replicates of experiments performed with
different larvae batches after 48 h (− (no induction); + (< 40%; low induction); ++ (> 40% high
induction).

Enrichment of outer membrane vesicles and mini cells from culture supernatants by
ultracentrifugation induced high transformation rates already within the first 24 h resulting in fully
transformed larvae after 48 h (entry 7). Thus, we reasoned that the morphogenic cues might be
part of the outer cell membrane and/or of high molecular weight. To determine the stability of the
morphogens and to optimize purification methods, we subjected the active HMW fractions (> 30
kDa) to enzymatic and physical treatments and tested the morphogenic response after treatment. As depicted in Figure 2, activity was completely retained when samples were treated with digestive enzymes such as DNase, RNase, or proteinase K, or even when heated to 96 °C for 10 min (entries 9-12). Treatment of the active fraction with aqueous 6 M NaOH or 6 M HCl (12 h, 30 °C) partially solubilized the active morphogen (entry 13, 14), and activity of both, soluble fraction and residue, was mostly retained after neutralization. However, treatment with 6 M HCl at higher temperatures (> 60 °C) gradually abolished the morphogenic activity of the sample. Based on these tests, we deduced that the morphogen(s) was neither a sensitive protein, nucleic acid nor an instable secreted metabolite. Morphogens were also extractable from the bacterial membranes and methanolic extracts showed indeed the highest morphogenic activity (entry 17, 18), while aqueous extracts retained only moderate activity (entry 15). Taken together, our results indicated that presumably two different bacterial morphogens are produced by P1-9, which are stable and extractable and likely localized within or at the bacterial outer membrane.

**Analysis of morphogenic phospholipids**

In a next step, we sought to characterize the morphogenic cues and applied a bioassay-guided reverse-phase column chromatography (HPLC) purification protocol. The resulting active HPLC fractions were analyzed by high-resolution tandem mass spectrometry (HR-MS²) and Global Natural Product Social Molecular Networking (GNPS) analysis revealing the dominant presence of different phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) species and the respective lyso derivatives (LPG, LPE). Due to inherent difficulties associated with the purification of structurally closely related phospholipids, we purchased eleven commercial derivatives that matched the proposed derivatives to compare the LC-HRMS² pattern (Figure S12-S17), as well as ^1^H and ^13^C nuclear magnetic resonance (NMR) spectra (chemical shifts and coupling constants) (S28-S52).

**Figure 3.** A) ^1^H-NMR spectra of commercial 16:0-18:1 PG (red) and morphogenic PG/LPG enriched fraction (blue). B-C) LC-HRMS² based GNPS analyses of purified lipid fraction showing the B) LPE and C) LPG MS²-cluster.
We then confirmed the morphogenic activity of commercial phospholipids including two
derivatives containing fluorescence labels (Figure 4) using 25 and 50 µM as final lipid
concentrations (based on preliminary dose-response tests using lipid extracts and commercial
16:0 LPG (Figure S15)). Here, it is noteworthy that phospholipid concentrations of single lipids or
combination thereof exceeding 50 µM occasionally caused the lysis of the organism (Figure 4).
Transformation rates were determined after 24 and 48 h, respectively; however statistical
analyses of assay data was not performed at this stage as induction by phospholipids resulted in
a non-synchronized behavior of larval transformation, similar to the observation made in axenic
biofilm assays. However, none of the tested single phospholipids recapitulated the full activity
observed for enriched phospholipid fractions derived from P1-9, but derivatives 16:0 LPG, 18:0
PE, and 16:0 (lyso)phosphatidic acid (16:0 LPA and 16:0 PA) repeatedly induced metamorphosis
in moderate to good rates (20-40%).
At this stage we questioned if the low solubility of (lyso)phospholipids might prevent the uptake
and perception by Hydractinia. Thus, we treated competent larvae with nitrobenzoxadiazole
(NBD)-labeled (phospho)lipids (18:1-12:0 NBD PG, 18:1-12:0 NBD PE or 12-NBD stearate
(Table S3), 25 nmol/mL) and found that all lipids were incorporated into the larvae membrane
within five hours and both phospholipid derivatives. While 12-NBD stearate did not induce
transformation to the primary polyp, 18:1-12:0 NBD PG and PE were found similarly active as
unmodified phospholipids (Figure 4). Here, it was noted that during metamorphosis the
fluorescence signal continuously decreased over time, presumably due to internalization and
decomposition of phospholipids during transformation processes (Figure 4, Table S3). Thus, it
was concluded at this stage that induction of metamorphosis by bacterial-derived phospholipids
presumably occurs by passive uptake of lipids into the larvae membrane, is strongly structure-
and concentration dependent and presumably orchestrated by Hydractinia-internal feedback-
loops.
Phospholipids (PL) and lysophospholipids (lyso-PLs, LPLs) are ubiquitous components of
bacterial cell walls and determine e.g. cell integrity and morphology as well as pathogen-host
interactions.29,30 However, little details are known about their possible signaling functions in
bacteria.31 As bacterial membranes and outer membrane vesicles (OMVs) are composed of
species-specific mixtures of (lyso)phospholipids, we hypothesized that the natural phospholipid
compositions of cells and bacterial biofilms will dictate the morphogenic activity and that additive
or even synergistic effects may play an important role in the induction process.
Figure 4. Metamorphosis inducing activity of commercial phospholipids. Transformation rates are given as averaged percentage ranges in 10% step (calculated from replicates of experiments performed with different larvae batches). Negative control: ASW; positive control: CsCl. A) Bioactivity test were performed using 25 and 50 µM final lipid concentrations. Transformation rates to morphological stage 9-11 and stage 12-14 were determined after 24 and 48 h. B-C) Activity tests of phospholipid combinations (25 µM of each lipid in a 1:1 combination with a final lipid concentration of 50 µM). Transformation rates to morphological stages 9-11 (B) and stages 12-14 (C) were determined after 48 h. D-K) Fluorescence labeling images of larvae treated with (D, E) 18:1-12:0-NBD PE; (F, G) 18:1-12:0-NBD PA; (H-I) 18:1-12:0 NBD PG undergoing metamorphosis (fluorescence imaging with emission wavelength at 527 nm).

Thus, we tested enriched phospholipid extracts (125 µg/mL lipid extract) of 16 different Hydactinia-associated bacterial strains including strains that were found inactive in biofilm assays for morphogenic activity (Figure S13). Indeed, extracts of several different strains (P1-23, SW13, P1-9, P. rubra, P3-9, P1-4-1a, P1-16) induced the transformation to the primary polyp (stage 12-14) in moderate to good rates within 48 h, in part exceeding the results of our previous biofilm experiments (Figure 1, S13). In contrast, lipid extracts of four strains caused cell lysis and/or abnormal transformations at the tested concentration, presumably due to the presence of overall toxic phospholipid concentrations or known cytotoxic (hydrophobic) natural products.
(violacein and/or poly-brominated pyrrole derivatives) naturally present within the bacterial cells and membranes of the producing organisms. We also analyzed the phospholipid composition of cell extracts by comparative high resolution tandem mass spectrometry (HRMS²) and found a species-specific abundance of phospholipids that only in part resembled the composition of our model strain P1-9. Despite the absence of detectable amounts of (L)PA derivatives, we hypothesized that the combination of several weakly active (L)PE and (L)PG might be responsible for the observed clear activity. To corroborate these findings, we then conducted combination assays of a defined subset of phospholipids (50 µM final concentrations). Indeed, several tested phospholipid combinations showed additive or even synergistic tendencies to induce metamorphosis within 48 h, in particular the combinations of 18:1 LPE/16:0 LPG, 18:1 LPE/18:1 LPG, 16:0 PA/16:0 PG, 16:0 LPA/16:0 LPG and 16:0 LPA/18:1 LPE (Figure 4). At this stage we concluded that specific (lyso) phospholipids and combinations thereof, known to be present in bacterial cell membranes and OMVs, are in part responsible for metamorphosis of Hydractinia larvae.

Analysis of morphogenic polysaccharide

As depicted in Figure 2, our bioassay-guided analysis indicated the presence of a second type of morphogenic compound of high-molecular weight (HMW, > 10kDa in size) and naturally present within aqueous extracts of Pseudoalteromonas P1-9 biofilms (Figure S9). Thus, we enriched for the secreted bioactive compound by size-exclusion filtration and analyzed the most active fractions by ¹H NMR, which revealed a complex mixture of yet unknown polysaccharides (Figure S21). Further purification (Sephadex G25, eluted with 0.1% NH₄OH) and semi-preparative HPLC (Shodex) and NMR and HRMS analysis of the most active fraction (> induction of 20%) revealed the presence of a polysaccharide consisting of repeating -(1′→4)-α-L-Rha-(1→3′)-D-Man- units (> 10 kDa, Figure 5, Figure S19-S27). The rhamnose/mannose composition was confirmed by acid hydrolysis using 6 M HCl, followed by TMS derivatization and GC-MS analysis and comparison with commercial standards (Figure S18). The purified polysaccharide, from now on named Rha-Man, showed a clear dose-dependent morphogenic induction of up to 80% transformation within 48 h (> 150 µg/mL, Figure S19). Partial acid hydrolysis resulted in loss of morphogenic activity. At this stage, we also tested rhamnose and glucose monomers at different concentrations, but no induction was observed. Comparative NMR-analysis of bioactive size-exclusion fractions revealed that Rha-Man was only a minor component of the highly bioactive polysaccharide fraction, which pointed towards additional not-yet structurally characterized P1-9-specific exopolysaccharides (EPS) derivatives with morphogenic properties (Figure S21).
Figure 5. Metamorphosis inducing activity of enriched and purified polysaccharides from P1-9 and commercial polysaccharides. Transformation rates are given as averaged percentage ranges in 10% step (calculated from replicates of experiments performed with different larvae batches). Negative control: ASW; positive control: CsCl. A) Bioactivity test were performed using (25 µg/mL) and transformation rates to morphological stage 9-11 and stage 12-14 were determined after 24 and 48 h, respectively. B - C) Bioactivity tests of phospholipids (10 µg per lipid) and curdlan combinations and 15 µg curdlan) were assessed after 24 h. D) Structures of repeating units of morphogenic polysaccharides.

Due to the inherent difficulties associated with the structural characterization of EPS in general and from P1-9 in particular, we decided to examine structure-function relations using structurally defined and more widely distributed bacterial polysaccharides (charged and non-charged). Intriguingly, curdlan, a well-known polysaccharide (50-200 kDa) with ß-1,3-glycosidic linkage and produced by the Gram-negative bacterium *Alcaligenes faecalis*, induced at significant lower dosage (25 µg/mL) the transformation to the primary polyp within 24 h (Figure 5). In contrast, paramylon, the 500 kDa derivative of curdlan, which differs in its average length and three-dimensional structure, induced the transformation only in moderate to low percentage. Other EPS derivatives, in particular charged EPS (hyaluronic acid and heparin), induced only in very low rates or were even toxic to larvae (Figure 5); secondary effects due to acidic/ionic properties cannot be excluded for these derivatives. At this step, we concluded that *Hydractinia* larvae respond in a dose-dependent fashion to the presence of two structurally distinct EPS derivatives and fully developed primary polyp (> 50% transformation rates) within 24-48 h.
We then tested if phospholipids and the identified polysaccharides induced morphogenesis in an additive or synergistic manner as both compound types co-occur within the bacterial biofilm. Thus, we tested combinations of moderately active lysophospholipids, 16:0 LPG and 18:1 LPE, as well as curdlan using lower concentration of individual compounds (10 and 15 µg/mL, respectively). As expected, curdlan showed dose-dependent activity and induced transformation to the primary polyp at lower rates compared to 25 and 50 µg/mL. Intriguingly, combinations of curdlan with either 16:0 LPG, or 18:1 LPE, or both derivatives resulted in almost 100% transformation rates within 24 h, which indicated towards synergistic (more than additive) activities. In addition, we also observed that larvae triggered by the combination of natural inducers developed more rapidly and cleanly into a fully functional primary polyp compared to artificial induction.

**Figure 6.** Specific phospholipids, naturally present in bacterial cells and outer membrane vesicles, and exopolysaccharides induce metamorphosis in *H. echinata*. A) Components of bacterial biofilms recruit and induce metamorphosis of competent *H. echinata* larvae; or prevent the transformation by killing larvae by secretion of toxic compounds. B) Scanning electron microscopy of single cells of P1-9 obtained from a three day old liquid culture. C-D) Negative contrast electron microscopic image of vesicles coated with S-layer like matrix and string-shaped biopolymers isolated from a three day old liquid culture of P1-9 (C) and agar plate derived P1-9 biofilm (D).
The lipophilic nature of phospholipids and insolubility of polysaccharides raised the question of how the bacterial signaling molecules are sensed and perceived by Hydractinia. In recent years evidence has been mounting that extracellular (or outer membrane) vesicles (OMVs) represent a highly conserved feature and potential mechanisms/vehicles of lipophilic signaling molecules that orchestrate the interaction between bacteria and marine eukaryotes.\textsuperscript{33-35} To assess if P1-9 produces OMVs, we performed electron microscopy (TEM) imaging of shaking and static cell cultures. Examinations of TEM images revealed entire cells and cells proliferating what appears to be outer membrane vesicles (100-300 nm, Figure 6). Interestingly, (cryo)TEM imaging of aqueous extracts of P1-9 biofilms and liquid culture revealed a high abundance of numerous extracellular vesicles of different sizes and forms (Figure 6A,C,D) as well as high abundances of biopolymer fibers (Figure 6B,C), which were positively tested for high morphogenic induction (Figure 2). Thus, it can be concluded at this stage that morphogens are naturally present within the biofilm matrix and are likely detected in form of OMVs and/or bacterial cell envelops.

Discussion
We successfully identified two types of bacterial morphogenic cues, a subset of (lyso)phospholipids and two bacterial polysaccharides (Rha-Man, curdlan) from gram-negative bacteria that induce the morphogenic transformation of Hydractinia planula larva into the primary polyp. Detailed structure-activity studies of both compound types revealed a strong concentration and structure-dependency of the morphogenic response. In case of phospholipids, 18:0 PE, 16:0 LPG and 16:0 LPA/PA showed the highest induction levels as single compounds and in combination with other lipids, while other phospholipid combinations were found to be inactive or even toxic to the larvae. Subsequent bioassays of phospholipid-rich bacterial extracts further indicated that the overall compositions dictates the final morphogenic response and effects could be overruled by the presence of toxic metabolites leading to abnormal or incomplete transformation or even death of the larvae. In case of polysaccharides, a P1-9 derived Rha-Man polysaccharide and A. faecalis-derived curdlan were found to induce the full transformation in a dose-response fashion with curdlan being the more active derivative. Intriguingly, combinations of phospholipids and curdlan induced the full morphogenic transition at rates exceeding in part the sum of single compound contributions and resulting in the complete transformation of almost all larvae within 24 h. Although, only representative derivatives of both bacterial compound classes were investigated in this study, the results are of crucial importance for future research directions in all facets for the following reasons:
Firstly, the structure, concentration and combination-dependency of the morphogenic response hints towards species-specific cross-kingdom interactions that might allow for the optimal habitat selection, despite the general global abundance of both compound classes in the bacterial world.\textsuperscript{36-38} Here, it can be speculated that only specific bacterial biofilm compositions, which induce the full transformation within less than 24 h, presumably enhance the likelihood of survival of the individual larvae in highly competitive environments. Thus, our study will guide future
investigations into the beneficial microbiome and the long-term survival of naturally-induced
Hydractinia colonies.
Secondly, our studies pave the way to examine additive and synergistic behavior of different
bacterial signals that trigger animal signaling systems. Only few studies have so far focused on
synergistic effects of morphogens in the marine environment,4-7 which includes studies on
sulfonolipids (RIFs) and LPEs, that in combination induce the formation of predatory rosette-like
stage of the choanoflagellate S. rosetta,39-41 and the recent identification of synergistically acting
nucleobases from marine bacteria, that induce metamorphosis of the invasive fouling mussel
Mytilopsis sallei.42 However, detailed mechanistic studies about mode of perception and
synergism in most model systems are thoroughly missing.
Thirdly, the identification of a novel polysaccharide and the detection of other morphogenic, yet
uncharacterized derivatives, encourages research directed towards the elucidation of their
biosynthetic pathways and regulation and might enable their future structure elucidation and
biosynthetic engineering.43-45
Fourthly, for Hydractinia it has been proposed from early on that neurosensory cells dominantly
located at or near the anterior pole and in part on its tapered posterior tip are responsible for the
detection of the bacterial cues.19,20 Upon physical contact with lipid-rich vesicles and cell
fragments naturally present within marine biofilms, phospholipids could passively integrate into
the Hydractinia membrane; a hypothesis that is supported by our findings that fluorescence-
labeled phospholipids are quickly integrated into membranes of Hydractinia larvae. Once
integrated, specific phospholipids could act as ligands for certain receptors or induce changes in
membrane fluidity resulting in the recruitment of, e.g., PKC involved in cellular signaling
processes.46-48 In addition, LPAs have been recognized as potent mitogen in humans since
decades due to their interactions with G-protein-coupled receptors (GPCRs), thereby altering
many different cellular responses in humans, such as proliferation, survival, cytoskeletal changes
or calcium influx.49 Thus, a homologous mode of action could be hypothesized for Hydractinia. In
contrast, morphogenic EPS molecules presumably require detection via dedicated receptors as
described for EPS-mediated host-pathogen interactions.50,51 In particular curdlan has been
previously reported to act on lectin-type receptors in humans; a receptor-type, which have also
been detected in a Hydractinia transcriptome study.52
In summary, the results of our studies will allow us to establish Hydractinia as a model system to
investigate more closely host-microbe interactions and will guide future research on molecule-
receptor interactions. Our results also open several avenues for future studies on other aspects of
how bacterial signals trigger animal development in the marine world, which could have potential
practical applications for preventing biofouling, coral reef management and aquaculture
husbandry.
**Materials and Methods**

Supplementary Information (SI) available: details on fermentation, cultivation, bioassays, isolation procedures, ESI-HRMS, $^1$H NMR, $^{13}$C NMR, and 2D NMR spectra as well as additional assay data.

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**Conflict of interest statement**

The authors declare no conflict of interest.
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