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Bacterial lipopolysaccharide induces settlement and metamorphosis in a marine larva.

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Abstract

Bacterially induced metamorphosis has been observed in marine invertebrate larvae from nearly every major marine phylum. Despite the widespread nature of this phenomenon the mechanism of this process remains poorly understood. The serpulid polychaete *Hydroides elegans* is a well-established model system for understanding bacteria-mediated larval development. A broad range of bacterial biofilm species elicit larval metamorphosis in this species via at least two mechanisms, including outer membrane vesicles and phage-tail bacteriocins. Here, we investigated the interaction between larvae of *H. elegans* and the inductive bacterium *Cellulophaga lytica*, which produces an abundance of OMVs but not phage-tail bacteriocins. We asked whether the OMVs of *C. lytica* induce larval settlement due to cell membrane components or through delivery of specific cargo. Employing a biochemical structure-function approach, and with a strong ecological focus, the cells and outer membrane vesicles produced by *C. lytica* were interrogated to determine the structure of the inductive molecule. Here we report that lipopolysaccharide is the inductive molecule produced by *C. lytica* that induces larvae of *H. elegans* to metamorphose. The widespread prevalence of LPS and its associated taxonomic and structural variability suggest that it could be a broadly employed cue to bacterially induced larval settlement of marine invertebrates.

Significance Statement

Whenever new surfaces are created in the sea, they are quickly populated by dense communities of invertebrate animals, whose establishment and maintenance requires site-specific settlement of larvae from the plankton. Although it is recognized that larvae selectively settle in sites where they can metamorphose and thrive and that the bacteria residing in biofilms on these surfaces are important suppliers of cues, the nature of the cues used to identify the ‘right places’ has remained enigmatic. In this paper, we reveal that lipopolysaccharide (LPS) molecules from a marine Gram-negative bacterium are the cuing molecules for a tropical marine worm and demonstrate the likelihood that LPS provides the variation necessary to be the settlement cue for the majority of bottom-living invertebrate animals.

Main Text

Introduction

The establishment and maintenance of benthic marine animal communities, from the intertidal to the greatest depths, depends on recruitment of larvae from the plankton. Larval recruitment is a critical process not only for all marine benthic communities, but also for creating harvestable populations of mariculture species like clams, oysters and shrimp. A critical element of this process is larval selection of habitats suitable for attachment, metamorphosis and growth in proximity to food and with opportunities to reproduce (1, 2). Understanding the processes that allow minute larvae, broadly dispersed in the ocean, to settle in the right places for survival, growth and reproduction remains a major question in marine biology.

In recent decades, we have learned that the cues larvae employ to select right places to metamorphose are almost universally biological, the majority originating from microbes residing in surface biofilms. Biofilm- or bacterial-induced settlement has been shown for larvae of sponges (3–5), cnidarians (6, 7), bryozoans (8, 9), molluscs (10–13), annelids (14), echinoderms (15, 16), crustaceans (17, 18) and urochordates (19). Although 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, we remain largely ignorant about the diversity of bacteria that stimulate larvae to settle, the structure of the bacterial cues, and the mechanisms involved in larval recognition of bacteria.

Larvae of the serpulid polychaete *Hydroides elegans* provide an excellent model for investigating bacteria-stimulated settlement (14, 20). *H. elegans* is a cosmopolitan member of the benthic and biofouling communities in warm-water bays and ports throughout the world (20). As is the case for a vast majority of marine invertebrates, an external stimulant induces an extremely rapid series of morphogenetic events that quickly transform the swimming larva of *H. elegans* into a bottom-living, tube-dwelling juvenile that will grow rapidly into a reproductive adult (20). Larvae of *H. elegans* typically do not settle in the absence of a biofilm, and this response follows direct contact with surface biofilms (14, 20–25). Investigations of the complex biofilm communities that induce settlement and metamorphosis in *H. elegans* and the cues that they produce must take place in the context of this ecology. Inductive cues must be naturally entrained within the biofilm and fast acting because even in a low turnover environment such as Pearl Harbor, current speeds and turbulence at the biofilm interface would otherwise rapidly disperse and remove any dissolved compounds (26–28).

Evidence has accumulated that it is particular bacterial species residing in the biofilms that induce the metamorphic response in the larvae of *H. elegans* (25, 29, 30). Inductive bacteria from a broad range of phyla, including both Gram-negative and Gram-positive strains, are inductive when cultured in mono-species biofilms (21, 25, 29, 31–33). However, some – probably most – biofilm-bacterial species do not induce settlement in *H. elegans* (25, 34). To date, we have identified at least two different induction mechanisms from two different Gram-negative bacteria, *Pseudoalteromonas luteoviolacea* and *Cellulophaga lytica.* For the bacterium *P. luteoviolacea* strain HI1, a highly inductive biofilm species isolated from Pearl Harbor, Hawaii and genetically characterized in our lab, we identified specific structural elements derived from phage-tail proteins that are in some manner involved in settlement induction (35–37). However, examination of other inductive bacterial species revealed that induction of larval settlement cannot alone be explained by phage-tail bacteriocins. *Cellulophaga lytica,* another biofilm bacterium, induces larvae of *H. elegans* to settle by an entirely different mechanism. Although cell-free preparations from broth cultures of *C. lytica* induce the tubeworm larvae to metamorphose, examination of the genome of *C. lytica* (38) yielded none of the genes that transcribe the phage-tail elements of bacteriocins(33). Transmission electron microscopy (TEM) of inductive supernatants from

C. lytica revealed an abundance of outer membrane vesicles (OMVs) and none of the bacteriocins found in similar preparations from *P. luteoviolacea* (33).

Gram-negative bacteria ubiquitously produce OMVs (39, 40), but their structures and contents are variable per species or strain; they may include toxins, genetic material, virulence factors and lipids (39). Consequently, OMVs provide the mechanism for a number of ecological roles fulfilled by Gram-negative species, including cell-to-cell signaling and transfer of DNA, proteins and small signaling molecules between cells (39, 40, 49, 41–48). We have previously suggested that such membrane vesicles should be considered for their potential to be a common mechanism of interaction between biofilm bacteria and invertebrate larvae (33). In this study, we asked whether the OMVs of *C. lytica* induce larval settlement because they are accessible pieces of cells membrane or because they deliver a specific cargo.

To gain key insight into the molecular structure of the larval settlement inducer produced by *C. lytica* and to determine if it is a protein, nucleic acid, or lipid, we subjected the OMV fractions to a battery of broad-spectrum enzymatic and chemical treatments and then tested the treated OMVs for loss of larval settlement-inducing activity. Subsequently, because OMVs are composed of pieces of the bacterial outer membrane and because of the need for larger quantities of compounds for treatments and assays, the components of the cell envelope (whole membrane, peptidoglycan and lipopolysaccharide) were assayed directly by extraction from whole cells. These approaches, including *in silico* analyses of bacterial genomes, methanol extractions of HPLC fractionation, and enzymatic destructions of OMVs and isolated cell membranes, combined with larval settlement assays, allowed us to reject all but the bacterial LPS as a cue for larval settlement. We conclude this is the key element in selective larval settlement by *H. elegans*, and we predict this will hold true for many other benthic marine invertebrate animals.

Materials and Methods

Larval culture and bioassays: Dioecious adult *H. elegans were* collected from docks in Pearl Harbor and maintained in the laboratory in continuously flowing, unfiltered seawater for several weeks without a noticeable decrease in fecundity. Gametes were obtained and larval cultured according to Nedved and Hadfield[18]. Briefly, worms were induced to spawn by removal from their tubes. Gametes were mixed. Fertilization and development proceeded rapidly to a feeding trochophore stage in one day. Larvae were fed the single-celled alga *Isochrysis galbana* Tahitian Strain at a concentration of approximately 60,000 cells.ml^{-1}. The larvae developed to the metamorphically competent nectochaete stage by day five and were utilized in metamorphosis experiments for 1-2 days. Because larvae of *H. elegans* will not metamorphose without exposure to bacterial biofilms or their products, wild, complex natural films developed on glass slides readily served as positive controls and maintaining the larvae in double filtered autoclaved seawater (DFASW) served as negative controls. Assays were carried out in 24-well plates, and the percent of larvae that metamorphose was determined at 20–24 hours. Significant differences ($p<0.05$) were calculated using Kruskal-Wallis followed by pairwise comparisons with false detection rate (FDR) correction(50). Raw data is available at https://dx.doi.org/10.6084/m9.figshare.10324772.

Bacterial culture and OMV isolation: *Cellulophaga lytica* was grown as previously described by Freckelton et al (33) on ½ FSWt media (51). Briefly, cryogen stocks were streaked onto ½ FSWt agar plates and incubated for 24-48 h at 25°C. Single colonies were then used to inoculate a 10 ml culture of ½ FSWt broth and incubated for 12 h at 25°C. An aliquot of this starter culture was then used to inoculate 200 ml cultures and incubated for a further 14 h at 25° C. Cells were harvested by low speed centrifugation (4000 *g*, 25 min 4°C). The cell-free broth was then filtered (0.2 µm, Millipore) to remove any remaining cells and then ultra-centrifuged (200,000 *g*, 2 h, 4°C) (33, 52) to isolate OMVs. All samples were examined by TEM using negatively stained preparations to confirm the presence of OMVs.

Growth phase analysis: Cultures of *C. lytica* were monitored for growth rate by measuring the OD_{600} of an aliquot hourly to determine the growth phase. This process was repeated with triplicate cultures and samples were collected at early log, late log and stationary phases. OMVs were isolated from each sample time point as described above and diluted to the same concentration in keeping with their FM-46-4 readings (Supplementary information).

Size exclusion analysis: Cell-free filtered (0.2 µm) conditioned media was used to isolate OMVs. OMVs from *C. lytica* were separated into size classes by ultrafiltration (Millipore, Centricon Plus-70, 3 kDa, 30 kDa, 100 kDa). 140 ml (2 x 70 ml) aliquots of conditioned media were applied to each ultrafilter and then centrifuged (4 000 *g* x 30 min x 4°C). Aliquots of the retentate and filtrate were taken for metamorphosis assays and TEM analysis. The remaining filtrate was then applied to the next ultrafilter (100 kDa > 30 kDa > 3 kDa).

Enzyme treatment of OMVs: Isolated OMVs were combined with enzymes (nucleases, proteases, lipases, lysozyme or trypsin) and incubated in a water bath at the optimal temperatures and time periods described in Table 1. Enzyme action was halted by heat treatment alone, no additional chemical inhibitors were added in order to avoid confounding interference with the larval response. For each enzyme or set of enzymes a seawater control was also performed to ensure that the responses observed were not a result of the enzymes or heat treatments alone. Successes of nuclease treatments were assessed by spectrometry and electrophoresis. Successes of protease treatments were assessed by spectrometry and SDS-PAGE.

Bioassay guided fractionation of *C. lytica*: Cell pellet (CLP) and cell free supernatant (CLS) and OMVs were extracted with organic solvents directly to identify metamorphosis-inducing molecules. CLP and OMV samples were extracted with methanol (MeOH) followed by a dichloromethane-methanol mixture (DCM: MeOH). CLS (3.5 L) was extracted by liquid: liquid partition with ethyl acetate (EtOAc). Each extract was subjected to reverse phase HPLC and all fractions were subjected to metamorphosis bioassay with larvae of *H. elegans*. For CLP, HPLC fractions were generated for bioactivity testing through an acetonitrile (ACN) gradient (10-25%, 20 mins; 25-40%, 10 mins; 40-100%, 20 mins; 100%, 10 mins). Active fractions were further purified using HPLC (Phenomenex, Luna, 5 µm, C18, 100 Å, 250 x 100 mm; MeOH 10%, 30 mins). For CLS, extracts were fractionated using a phenyl hexyl column (Phenomenex, Luna, 5 µm, Phenyl-Hexyl, 250 x 100 mm) and a water acetonitrile gradient. Active fractions underwent ^{1}H NMR and HR-MS analysis. Specific purification details of isolated compounds are in the supplementary materials.

In silico analysis for the presence of secondary metabolites in the genome of *C. lytica*: AntiSMASH and NaPDos are bioinformatic platforms that allow genome mining for secondary metabolite biosynthesis gene clusters in bacterial genomes (53, 54) and allow rapid genome-wide identification of secondary metabolites genes, C- or KS- domains or amino acids. An *in silico* analysis of the genome of *C. lytica* (CP009239.1) using both AntiSMASH 5.0 (53, 54) and NaPDos (55) were run with default, relaxed parameters to determine the potential for *C. lytica* to produce secondary metabolites (Tables S3-5).

Extraction of *C. lytica* cell-envelope component: **Cell Membrane** Whole cells of *C. lytica* (500 µl) recovered from broth culture were sonicated on ice (4 x 15 s, 200 W), the samples were centrifuged for 5 mins at 10000 *g.* A total of 3 cycles were completed. When no more cells pelleted, the sample was ultra-centrifuged (45,000 *g*, 4°C, 1 h) to pellet the insoluble components of the membrane. The membrane sample was resuspended (100 µl) in double filtered autoclaved seawater (DFASW) and serially diluted for bioassay.

Extraction of *C. lytica* cell-envelope component: **Lipopolysaccharide.** Lipopolysaccharides (LPS) was extracted using the Westphal hot phenol method (56) as updated by Apicella et al. (57) and

serially diluted (0.1, 0.01, and 0.001 X) for assessment of metamorphic induction in larvae of *H. elegans*.

Bioactivity testing of commercially available cell envelope components: **Peptidoglycan Monomers**. Muramyl dipeptides and muramyl tripeptides (Sigma Aldrich) were tested for induction of metamorphosis in larvae of *H. elegans* individually across a range of concentrations (1–100 µM) by dissolving in H_2O (MilliQ). Additionally, a solution containing 10 µM concentrations of both compounds was also screened for metamorphic induction.

Examination of bacterial preparations with transmission electron microscopy (TEM): Bacterial preparations and cell-free preparations were applied to glow-discharged formvar grids, negatively stained with 2% uranyl acetate aqueous solution and air dried. Samples were imaged using a 120 kV Hitachi HT7700 with an AMT XR-41.

Results

OMV production, size and metamorphic bioactivity as a function of growth phase in *C. lytica*:
OMVs isolated from broth cultures of *C. lytica* at mid-log, late log and stationary phase were tested for their ability to induce metamorphosis in the larvae of *H. elegans*. While OMVs isolated at each of these stages were bioactive, average larval metamorphosis of *H. elegans* was highest from OMVs isolated during stationary phase and lowest from mid-log phase OMVs (Figure 1). Notably, the abundance of vesicles was also highest during stationary phase (Figure 1). In addition, because the OMVs vary in size, we separated them into size classes (<3 kDa, 3-30 kDa, 30-100 kDa) by filtration and assayed them for their ability to trigger metamorphosis in larvae of *H. elegans*. We found no significant differences in bioactivity from OMVs between size classes (Figure 1C). Importantly, all growth phases and OMV size classes induced metamorphosis in larvae of *H. elegans* (Figure 1).

Enzymatic interrogations of OMVs for larval metamorphic cues: Nucleic acids and proteins
The OMVs from *C. lytica* were interrogated enzymatically to determine whether larval settlement induction was due to the presence of a nucleic acid, protein cargo or to the membrane component. Treatment with nucleases (DNase I and RNase) had no impact on the metamorphosis-inducing capacity of the OMVs (Figure 2A). Similarly, when OMVs were treated with Proteinase K or Pronase E there was no impact on the induction of metamorphosis by the OMVs (Figure 2A). The absence of nucleic acids in the OMV samples was confirmed by electrophoresis and UV absorbance, and the enzymatic destruction of proteins was confirmed by SDS-PAGE. Interestingly, both proteases had to be removed from the treatment by ultracentrifugation prior to larval assay, because the residual enzymes themselves induced metamorphosis (Fig S1).

Enzymatic interrogations of OMVs for larval metamorphic cues: Lipids
We next focused our investigations on studies of the OMV membranes and lipid-based cargo. Treatment with lipases and phospholipases significantly decreased OMV induction of metamorphosis (Figure 2B). Metamorphosis after OMV treatment with Phospholipase A_1, while still significantly higher than the treatments with all other lipases, was significantly lower than the untreated OMV and positive controls ($p<0.05$; Figure 2B). No molecular structure-activity relationship for the metamorphic cue can be predicted from the difference in reduction of metamorphosis between the phospholipase A_1 and A_2 treatments (Figure 2B). Phospholipase A_2 is composed mostly of linear sheets and consequently will be subject to less steric hindrance than the barrel shaped phospholipase A_1 (58). These results indicate the involvement of a bioactive lipid.

Enzymatic interrogations of OMVs for larval metamorphic cues: peptidoglycan
Because it has been shown to be an active component of bacterial signaling in other systems (59), the possibility that peptidoglycan is the source of settlement-induction activity was assayed with

two separate techniques: (1) treatment of bacterial OMVs with lysozyme, followed by bioassay (Figure 3) and; (2) direct larval assays with commercial muramyl peptides. Commercial muramyl dipeptides and muramyl tripeptides, tested across a concentration range of 1–100 µM, resulted in no metamorphosis of larvae of *H. elegans* when tested separately or in combination. Similarly, the lysozyme treatment of the OMVs had no impact on metamorphosis of larvae of *H. elegans* (Figure 3).

Isolation and identification of secondary metabolites of *C. lytica* for induction of larval metamorphic cues:

In order to assess whether the metamorphosis induced in larvae of *H. elegans* by OMV's from *C. lytica* was due to an inherent component of the membrane or a small molecule encapsulated as cargo, a bioassay guided fractionation approach to isolate and identify bioactive small molecules, as well as an *in silico* genomic assessment for the presence of secondary metabolite genes were conducted.

Isolated OMVs and whole (cultured) cells were subjected to bioassay-guided fractionation to ensure agreement with the previously observed larval induction activity. To increase the amount of membrane materials for chemical and bioassay analyses, we turned to utilizing large quantities of whole bacterial cells. Amino acids (phenylalanine and leucine), niacin, thymidine, adenosine, and a 2,5-diketopiperazine of phenylalanine and proline were isolated from moderately active HPLC fractions of the extracted cells of *C. lytica* (Figures S2-5). To confirm the identities of the isolated compounds, commercial versions of the compounds were also tested. Neither extracted amino acids, nor their commercial counterparts induced more than 37% metamorphosis (Figure 4). It should be noted that induction of larval metamorphosis did not begin until after more than 6 h of exposure to any of the fractions, which is exceptionally slow.

Two genome database search engines (AntiSMASH and NaPDos) were employed to identify potential secondary metabolite genes or domains in the genome of *C. lytica*. AntiSMASH analyzes bacterial genomes for secondary metabolite-biosynthesis gene clusters (53, 54), and NaPDos is designed to detect and extract condensation (C-) and ketosynthase (KS-) domains from DNA (55). AntiSMASH analysis revealed one potential region of secondary metabolite significance from 445 027–465 863 bp; however, this region had only a 28% match to a terpene (Tables S1-3). This region most likely encodes a carotenoid, which may impart the yellow pigment of the bacterium. No other regions of interest were identified. Likewise, the NaPDos database revealed two KS domains, however, with 55% or less identity indicating a poor match.

Analysis of cell envelope and lipopolysaccharide for larval metamorphic cues:

Because lipases significantly reduced settlement induction and the absence of a lipophilic small molecule in the natural products isolations, our focus returned to the lipophilic components of the cell envelope of *C. lytica*. Settlement experiments using pieces of cell membrane acquired through repeat sonication and ultracentrifugation of cells were inconclusive. This is because high concentrations of the membrane resulted in death and lysis of the larvae, but low concentrations had no effect on the larvae. The remaining major lipophilic component of the bacterial membrane, lipopolysaccharide, was next evaluated. Extracted and purified LPS successfully induced metamorphosis in the larvae of *H. elegans* (62%; Figure 5 in under an hour. The absence of peptidoglycan and proteins from this preparation was confirmed using SDS-PAGE, comparing Coomassie blue staining with silver staining.

Discussion

The broadly distributed marine polychaete *H. elegans* serves as a useful proxy for undoubtedly thousands of benthic marine invertebrate species whose larvae settle and metamorphose in response to specific biofilm-dwelling bacteria (4, 9, 20, 21, 33, 60–63). However, it can be difficult to know whether bioactive chemicals identified in the laboratory are the same as those that act *in*

situ or simply stimulate processes downstream of the receptor (64–68). The practice of isolating and identifying compounds involves the exposure of the larva to substances with which it may not naturally interact (64). While experimentally valuable, artificial induction of larval metamorphosis does not enable us to learn more of the true induction of metamorphosis that is so essential to benthic marine organisms. Fortunately, the wealth of knowledge accumulated for larval settlement conditions provides sound criteria for the acceptance or rejection of the ecological relevance of any isolated cue. Our conditions for acceptance of an ecologically relevant cue to induce metamorphosis in *H. elegans* require it to be bacterial in origin, fast acting and entrained to the biofilm (23). It was with this focus that we asked the question: what chemical component of the OMVs from *C. lytica* cues the larva of *H. elegans* to settle.

OMVs can be considered as a secretion or delivery system and are a key component for bacterial interactions with the environment (40, 69). Previous studies have shown that OMVs produced at different culture growth stages or of different sizes can vary in composition and biological activity (40, 69, 70). Our data, however, demonstrated bioactivity in all growth stages, with a steady but non-significant increase over time, suggesting that the bioactive compound produced by *C. lytica* may be either constitutively produced or has a long half-life and may proportionately increase in cultures over time.

To determine the nature of the bioactive compound(s), we subjected *C. lytica* OMV fractions to a battery of enzymatic and chemical treatments before assaying these fractions for loss of larval settlement-inducing activity. Enzymatic interrogations of the OMVs strongly indicated that induction of metamorphosis was not due to their delivery of nucleic acid or protein cargo. These observations, in combination with the discovery that, in culture, OMVs were active settlement inducers regardless of their sizes or when they were produced, suggested that OMVs induced metamorphosis because they were small pieces of cell membranes, rather than because they were specifically secreted as a delivery system. For this reason, we next focused our investigations on studies of the OMV membranes.

The involvement of a lipophilic molecule was implicated by the reduced induction of metamorphosis when OMVs from *C. lytica* were treated with lipases. These results indicate the involvement of a phospholipid or other bioactive lipid. Phospholipids are not only essential components of all cell membranes but are also important and widespread intracellular (second) messengers (71). Although molecules that act as intracellular messengers are unlikely to be the cue from *C. lytica* that induces metamorphosis in larvae of *H. elegans*, a small number of phospholipid metabolites have been identified as intercellular (primary) messenger molecules (71). Currently all of the identified intercellular messenger molecules of this class are small mono-acyl phospholipids or lysophospholipids (71), which are produced by selective hydrolysis of phospholipids by phospholipases (71). Consequently, if phospholipids and their metabolites are responsible for the induction of larval metamorphosis, greater induction of metamorphosis would be expected as a result of phospholipase treatment of OMVs. Instead, the phospholipase treatment of OMVs significantly reduced induction of metamorphosis suggesting that neither phospholipids nor their metabolites are not involved. The non-involvement of phospholipids is further supported by previous work by Holm et al. (24), who determined that G-protein coupled receptors, the main receptors for these phospholipid pathways, were not involved in triggering the metamorphic cascade in *H. elegans*.

Elimination of phospholipids as the bioactive component of the OMVs affected by the lipase treatments leaves two additional avenues of interpretation. Firstly, the results of the lipase treatments of OMVs could be due to the presence of a bioactive non-phospholipid, and the reduction in metamorphosis observed might be due to non-specific enzyme action. Secondly, the reduction in larval metamorphosis as a result of the lipase treatments may reflect the requirement for an intact membrane or OMV; i.e., the action of lipases destroys the structural integrity of the membrane. If so, any inductive cue that requires delivery by the OMV or the cell itself would be

rendered inactive when it was no longer delivered. This would also be true of any compound associated with the outer membrane that required a particular conformation provided by the membrane in order to interact with the larvae. Each of these possibilities is examined here.

Because it has been shown to be an active component of bacterial signaling in other systems (59), the peptidoglycan cell wall was next examined for the capacity to induce larval settlement. The macromolecule peptidoglycan forms a mesh-like layer between the plasma membrane and the outer-membrane in Gram-negative bacteria and provides rigidity and shape to bacterial cells. This mesh lattice is the result of polymerization of monomers consisting of the amino sugars *N*-acetylglucosamine (GlcNAc or NAGA) and *N*-acetylmuramic acid (MurNAc or NAMA) by β-(1,4)-glycosidic bonds (72). Additionally, each MurNAc is attached to a short amino acid side chain. Although occurring beneath the outer-membrane layer, peptidoglycan fragments can be included within OMVs if the layer is disrupted. Results of larval assays to OMVs treated with lysozyme and commercially available components of bacterial peptidoglycan were negative. Although bioactive peptidoglycan has been found in other systems (59, 73), our result is not entirely surprising since the peptidoglycan layer resides beneath the outer membrane and would not be the first point of interaction between a larva and a bacterium or an OMV.

The presence of a bioactive lipid, mono-acyl phospholipid or other small molecules as the source of the larval metamorphosis inductive cue was further investigated and rejected using both bioassay guided fractionation and *in silico* approaches. Methanol extractions of OMV preparations followed by HPLC separation produced mostly common small molecules such as, amino acids. Induction of metamorphosis by dissolved free amino acids (DFAAs) has been investigated previously (74–76) but was determined unlikely to have ecological relevance. This doubt was due to delayed onset of metamorphosis, low levels of metamorphosis, undetectability of DFAAs in field studies and the presence of bacterial biofilms in the experimental set up (74–76). Our results agree with these conclusions, when we tested amino acids, metamorphosis was rare and delayed.

Induction of metamorphosis can be stimulated in larvae of *H. elegans* by artificial inducers, such as an excess of potassium or cesium ions (22, 65, 66, 68) and other non-relevant compounds (75). This knowledge combined with the non-specificity of amino acids strongly indicates they are unlikely to be the true inducer produced by *C. lytica*. Certainly, amino acids cannot explain the selective choices of settlement locations observed in the wild or the requirement for physical contact between the larvae and the biofilm (23). For these reasons, we concluded that none of the low-activity metabolites from the bioassay guided fractionations were involved in natural metamorphic induction.

Genomic mining for secondary metabolite gene clusters is an increasingly important tool for identifying of bioactive molecules (77). The successful employment of these techniques relies upon the completeness and correctness of secondary metabolite gene annotations and databases (77); while such databases are improving daily they remain incomplete (53, 78, 79). Furthermore the presence of secondary metabolite gene clusters in an organism's genome cannot alone determine the presence of the encoded compound but rather the potential for production of that compound (53, 78, 79). What we found when we examined the genome of *C. lytica* with both AntiSMASH and NaPDos pipelines was inconclusive. Importantly, however, these *in silico* searches confirmed the results from bioassay guided fractionation approach; no gene clusters or domains were identified that could transcribe compounds likely to induce complex larval metamorphosis.

Because lipases significantly reduced settlement induction and the lack of a lipophilic small molecule in the natural products isolations, our focus returned to the lipophilic components of the cell envelope of *C. lytica*. The remaining major lipophilic component of the bacterial membrane, LPS, would be the first bacterial molecule to interact with a larva upon contact.

Lipopolysaccharide (LPS) is located on the outside of OMVs as well as whole cells and is thus consistent with the observed activity for OMVs from *C. lytica*. In other words, the discovery that bacterial LPS provides the inductive cue for settlement and metamorphosis of larvae of *H. elegans* is consistent with all the observations made previously and in this study.

Interactions between microbes and eukaryotes are mediated largely by a suite of conserved molecules knowns as Microbe-Associated Molecular Patterns or MAMPs, of which LPS is a prototypical example (80, 81). The ubiquity of a MAMP such as LPS, means that the response to its detection by a eukaryote is entirely context dependent. Indeed, MAMPs are implicated in both well-established symbiotic relationships, as well as, pathogenic ones (82). The strong immunogenicity of LPS, combined with its ubiquitous expression by Gram-negative bacteria, make it both a likely target of evolutionarily conserved innate-immune receptors in larvae and suggest a possible wide-spread mechanism for bacterial induction of marine-invertebrate metamorphosis. Furthermore, LPS is a highly variable macromolecule with variability tied closely to bacterial taxonomic lines and growth conditions (83–87).

Structural variability of LPS provides a mechanism to explain variation in bacterial induction and suggests that LPS is responsible for induction of metamorphosis in a broad swathe of marine invertebrates in specific habitats. LPS is composed of three main components: lipid A (endotoxin); an inner core oligosaccharide (kDO); and an outer core oligosaccharide (o-antigen) with variation occurring primarily in the lipid A and o-antigen components. If variations in the structure of LPS are responsible for variable inductive activity of different species and strains of bacteria, it explains why many – but definitely not all – marine Gram-negative bacteria induce settlement and metamorphosis in the larvae of *H. elegans* and other invertebrate species, and why not all strains of the same bacterium induce larvae of a single invertebrate species to settle[32]. Most importantly, it explains the differential settlement of different invertebrates in different habitats. *H. elegans* is a cosmopolitan species, and its larvae respond to many different, but definitely not all, bacterial species (14, 20, 25). LPS and its inherent variation could explain this phenomenon, raising the possibility that LPS is the metamorphic cue in many inductive bacterial species.

While LPS may largely explain the widespread occurrence of inductive activity across Gram-negative biofilm bacteria, it cannot account for the same activity found in Gram-positive bacteria (33). The cell envelope of Gram-positive bacteria differs greatly from that of Gram-negative bacteria, lacking both an outer membrane and its LPS element. Instead, Gram-positive bacteria have a thickened cell wall of peptidoglycan that can be populated by teichoic and lipoteichoic acids (LTA) (88, 89). LTA is comprised of a glycolipid and a hydrophilic polymer of glycerophosphate covalently bound to the former. LTA has been determined to be a component of nearly all Gram-positive membranes, *as well as a functional equivalent to LPS*[31]. Thus, we predict that LTA from the inductive Gram-positive bacteria will be the active moiety in induction of metamorphosis.

Conclusions and Implications

The broadly distributed marine polychaete *H. elegans* serves as an excellent model organism for benthic marine invertebrate animals whose larvae settle and metamorphose in response to specific biofilm-dwelling bacteria. We had previously learned that larvae of *H. elegans* will settle only after contacting complex natural biofilms or monospecific films formed by isolates from those biofilms. We knew, also, that larvae of *H. elegans* will settle in response to outer membrane vesicles isolated from broth cultures of the bacterium *Cellulophaga lytica*. LPS satisfies these ecological criteria for acceptance as an inductive cue, namely, it is bacterial in origin, entrained to the biofilm and fast-acting. Furthermore, the widespread distribution of this molecule and its taxonomic-associated variability, position it well to explain not only the selective settlement of *H. elegans* but potentially many other benthic invertebrates. The widespread nature of LPS and

its associated taxonomic and structural variability suggest that it could be a broadly applicable cue to bacterially induced larval settlement of marine invertebrates.

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Figures and Tables

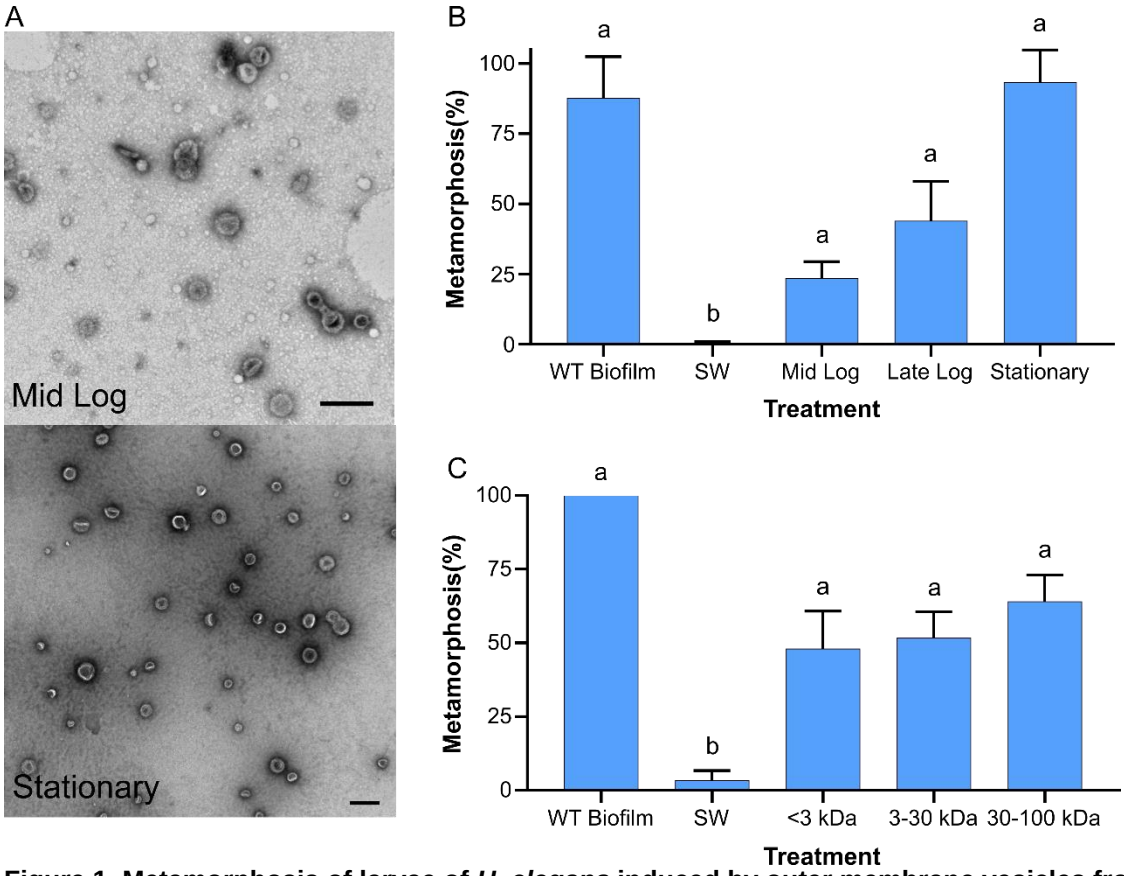

Figure 1. Metamorphosis of larvae of *H. elegans* induced by outer membrane vesicles from *C. lytica*. (A) Negatively stained TEM image of OMVs from the mid log and stationary stage of a culture of *C. lytica*. Scale bar 200 nm. (B) Metamorphosis of larvae of *H. elegans* when exposed to OMVs from each growth phase (Mid Log, Late Log and Stationary). (C) Metamorphosis of larvae of *H. elegans* when exposed to OMVs from different size classes. Metamorphosis was counted after 24 h exposure, with filtered seawater serving as a negative control, and a multispecies biofilm serving as a positive control. Letters show significant differences.

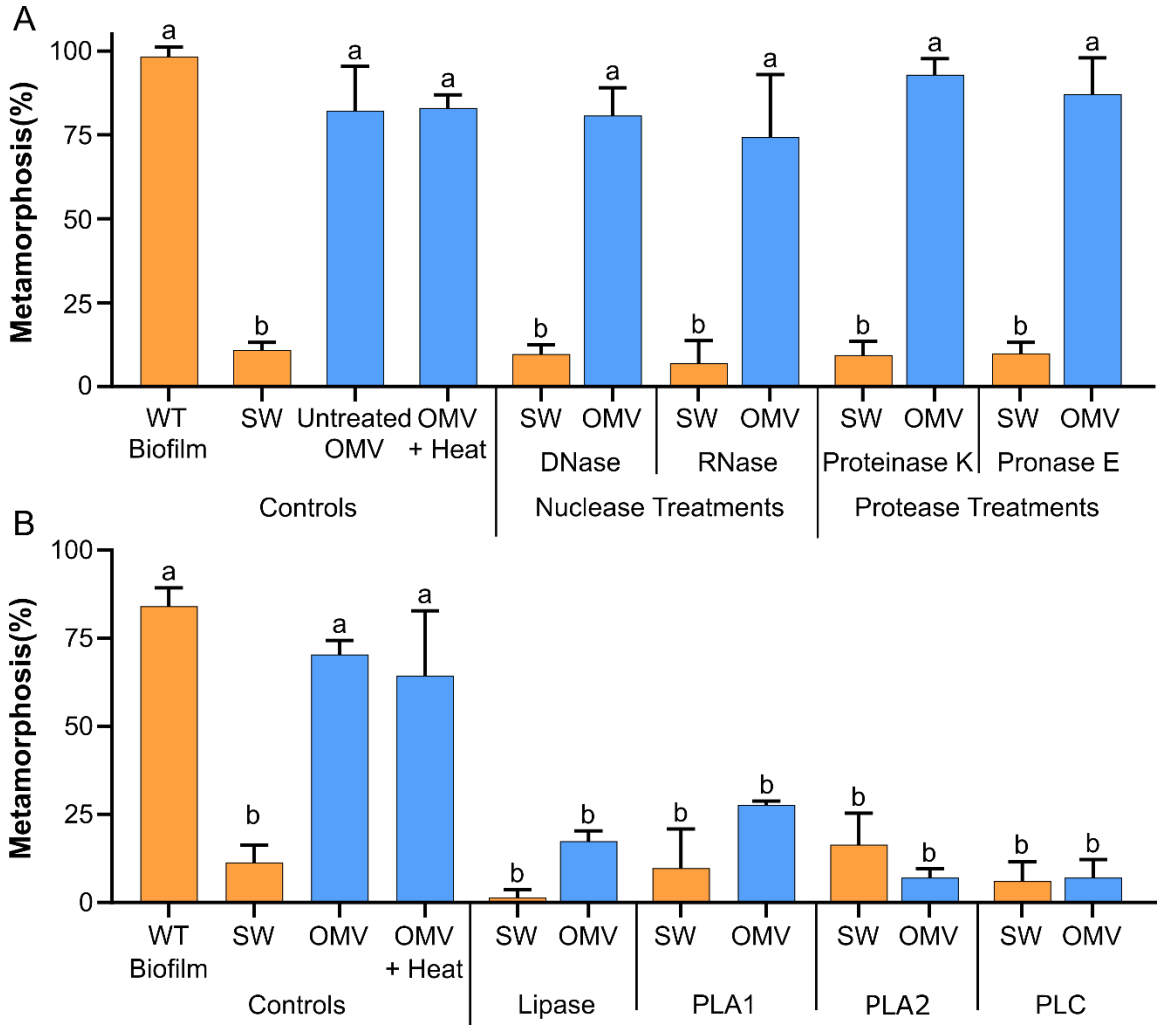

Figure 2. Induction of metamorphosis in larvae of H. elegans from enzyme treated outer membrane vesicles (OMVs) from *C. lytica*. (**A**) Nucleases (OMVs and seawater (SW) controls were exposed to either DNase (200 U) or RNase (200 U) and Proteases (OMVs and SW controls were exposed to trypsin (20 µg/mL) followed by either Proteinase K or Pronase E (100 µg/ml)). (**B**) lipase, phospholipase A_1 (PLA1), phospholipase A_2 (PLA2) and phospholipase C (PLC). Metamorphosis was counted after 24 h exposure. Filtered seawater (SW) served as a negative control, and a multispecies biofilm and an untreated OMV were used as positive controls. Letters indicate significant differences.

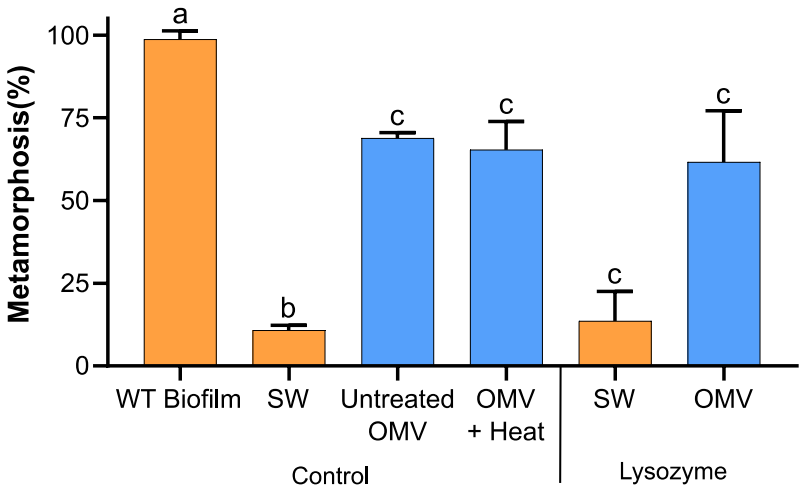

Figure 3. Treatment of OMVs with lysozyme did not impact their capacity to induce settlement. Metamorphosis of larvae of *H. elegans* was counted after 24 h exposure to outer membrane vesicles treated with lysozyme (50 U). Negative control: filtered seawater (SW), positive control: Wild type (WT) biofilm and an untreated OMV preparation from *C. lytica*. Letters indicate significant differences.

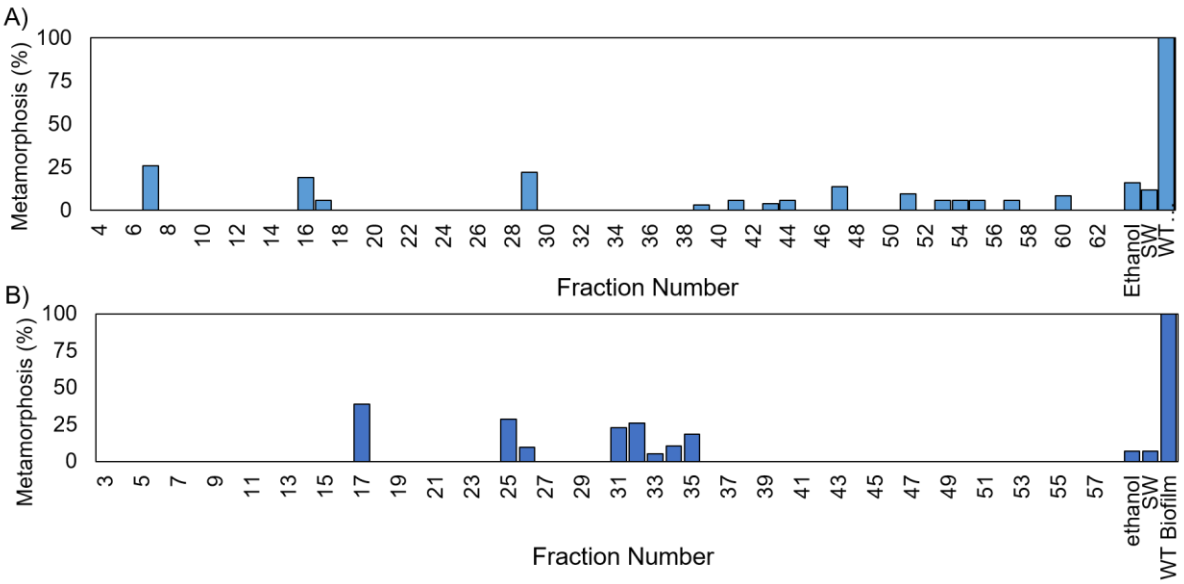

Figure 4. Metamorphosis induction in larvae of *H. elegans* was consistently low from methanol extracts (10 µg/ml) of A) cell pellet; B) cell-free culture supernatant containing outer membrane vesicles (OMVs) of *C. lytica*. Extracts were solubilized in ethanol, transferred to wells, evaporated to remove solvent and sterile seawater and larvae were added. Solvent control: ethanol, negative control: sterile seawater (SW), positive control: Wild type (WT) biofilm.

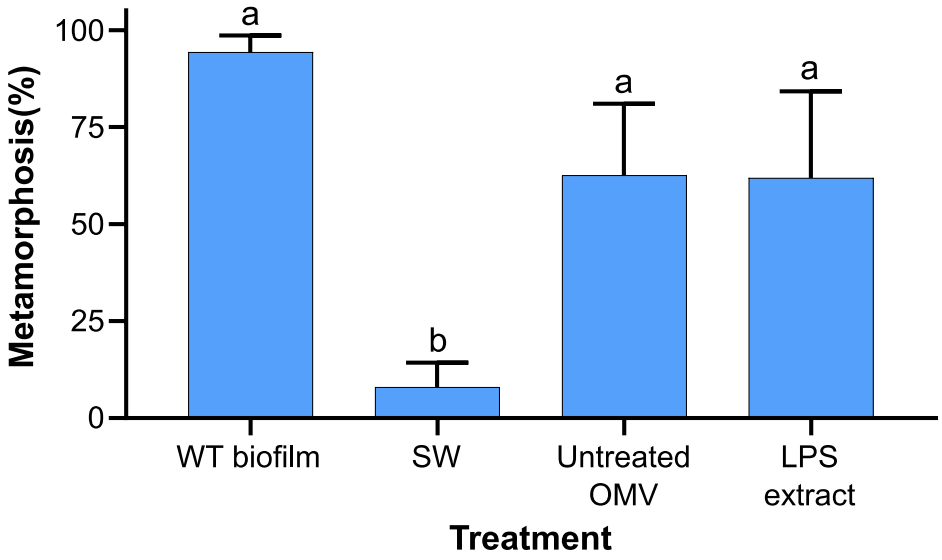

Figure 5. Lipopolysaccharide, isolated using the hot phenol method, induced metamorphosis in the larvae of *H. elegans.* Metamorphosis was counted 24 h after exposure. Negative control: sterile seawater (SW); positive control a wild type (WT) biofilm.

Table 1: Enzyme treatments of OMVs from *C. lytica*

Cell component	Enzyme	Incubation			Thermal Inactivation	
		Conc	Time	°C	°C	Time
Nucleic acid	DNAse	200 U	2.5 h	37°C	75°C	10 min
	RNAse	200 U	2.5 h	37°C	75°C	10 min
Proteins	Proteinase K	100 µg/ml	2.5 h	55°C	100°C	10 min
	Pronase E	100 µg/ml	2.5 h	55°C	100°C	10 min
	Trypsin	20 µg/ml	2.5 h	37°C	100°C	10 min
Peptidoglycan	Lysozyme	50 U	2.5 h	37°C	100°C	10 min
Lipid	Lipase	100 U	2.5 h	55°C	100°C	10 min
	Phospholipase A_1	100 U	2.5 h	55°C	100°C	10 min
	Phospholipase A_2	100 U	2.5 h	55°C	100°C	10 min
	Phospholipase C	100 U	2.5 h	55°C	100°C	10 min