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Antagonism of Bacterial Extracellular Metabolites to Freshwater-Fouling Invertebrate Zebra Mussels, *Dreissena polymorpha*

Ji-Dong Gu[1]* and Ralph Mitchell

Laboratory of Microbial Ecology, Division of Engineering and Applied Sciences, Harvard University, 40 Oxford Street, Cambridge, Massachusetts 02138, USA

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We investigated the antagonism of indigenous bacteria isolated from stressed mussels and their extracellular metabolites on the adult zebra mussel, *Dreissena polymorpha*. Selective bacterial isolates including *Aeromonas media*, *A. salmonicida*, *A. veronii*, and *Shewanella putrefaciens*, showed strong lethality against adult mussels and 100% mortality was observed within 5 days of incubation. Bacterial metabolites, fractionated and concentrated from stationary-phase culture supernatants of these bacterial isolates, displayed varying degrees of antagonistic effects on zebra mussels. Among the three size fractions examined, <5, 5-10, and >10 kDa, the most lethal fraction seems to be >10 kDa for three of the four isolates tested. Further chemical analyses of these size fractions revealed that the predominant constituents were polysaccharides and proteins. No 2-keto-3-deoxyoctanoic acid (2-KDO), deoxyribonucleic acids (DNA) or uronic acid were detectable. Extraction of supernatants of two antagonistic isolates with polar solvent suggested that polar molecules are present in the active fraction. Our data suggest that extracellular metabolites produced by antagonistic bacteria are also involved in disease development in zebra mussels and elucidation of the mechanisms involved may offer a novel strategy for control of biofouling invertebrates.

Key words: zebra mussels, *Dreissena polymorpha*, biological control, antagonism, bacterial isolate, metabolites, mortality

The spread of the zebra mussel, *Dreissena polymorpha*, in the freshwaters of North America has resulted in significant impacts on the ecology and economy of the infested regions. This biological invader is believed to have been first introduced by ballast water from trans-Atlantic vessels into the Laurentian Great Lakes (4, 15). Since then, mussels have successfully adapted to the waters of North America without encountering known predators or parasites so far. The increase of the zebra mussel population has resulted in a decline of native filter-feeding species of bivalves, e.g., *Lampsilis siliquodea* (14) because mussels consume the primary producers phytoplankton which serve as a food source for other aquatic animals. Economic losses are also directly reflected in biofouling of the water intakes of treatment facilities and utility cooling towers (19, 21) as well as in the aquaculture industy (6).

Current control strategies include chemical treatment using chlorine and biocides to eradicate the fouling problem (27). An intensive physical cleaning effort is usually required to detach the byssal threads of mussels from substratum surfaces. The use of electrical current (33) and magnetic systems (34) have also been proposed as means to combat adhesion by the mussels. However, little is known about the relationship between natural and indigenous microbial pathogens and the host animals in this new environment. Biological control of mussels using indigenous opportunistic microorganisms may be a realistic alternative controlling strategy if a better understanding can be achieved (9-12).

We have demonstrated that opportunistic pathogens can be isolated from tissue of *D. polymorpha* and used in killing adult mussels effectively (12). The dead mussels displayed severe tissue damage. Based on our observations, we suspect that bacteria and their extracellular metabolites may be involved in the mortality of mussels (12). The objective of the current study was to demonstrate the role of extracellular metabolites antagonistic to mussels.

[1]Present address: Department of Ecology & Biodiversity, and the Swire Institute of Marine Science, Kadoorie Biological Sciences Building, The University of Hong Kang, Pokfulam Road, Hong Kong SAR; and Environmental and Molecular Microbiology, South China Sea Institute of Oceanography, Chinese Academy of Sciences, 164 West Xinggang Road, Guangzhou 510301, P.R. China
* To whom correspondence should be addressed.
(Tel) 852-2299-0605; (Fax) 852-2517-6082
(E-mail) jdgu@hkucc.hku.hk

Materials and Methods

Test animals

Zebra mussels from Lake Ontario were used exclusively in our experiments. The mussels were obtained by divers and transported overnight on ice to the laboratory at Cambridge, Massachusetts. Upon arrival, they were immediately immersed in artificial lake water (ALW) (10% Instant Ocean in distilled water) (Instant Ocean, Aquarium Systems Inc., Eastlake, OH, USA) in an aquarium (80 L internal capacity), which was aerated by an airpump. The aquarium was housed in a temperature-controlled incubator at 10°C. No feeding was necessary during storage for a maximum of one year.

Tests for antagonism

Bacterial isolates (HU-Z1, Z2, Z3, and Z4), previously isolated and preserved on Plate Count Agar (PCA) plates or PCA slants (12), were used to inoculate Erlenmeyer flasks containing 100 mL nutrient broth medium (Difco Lab., Detroit, MI, USA), and the flasks were incubated in a water bath at 26°C until the bacteria reached the mid-log phase. Then bacteria were harvested by high speed centrifugation at 10,000 × *g* for 15 min, and the supernatant was discarded. The cell pellet was resuspended in 100 mL 0.0375 *M* phosphate buffer plus 0.1% (w/v) peptone (Difco Lab., Detroit, MI, USA) and recentrifuged. The supernatant was again discarded and the washing process was performed twice. The bacterial cell pellet was suspended in 50 mL of the same phosphate buffer solution and cell density was enumerated by acridine orange direct count (AODC) using epifluorescence microscopy (16). Appropriate dilutions were made based on the AODC counts to achieve a projected cell density of 10^9 cells mL^{-1} for inoculation of zebra mussels.

Juvenile zebra mussels between 9-15 mm were taken out of the aquarium and placed in sterile Petri dishes. At least twenty mussels were initially used in each treatment. The animals were first conditioned to room temperature for about 6 hr, then placed under a sterile hood for 2 hr to allow the external water to evaporate. They were then injected with 10 μL of bacterial suspension in the abdominal side at about a 30 degree angle to the rear. The cell concentration of the inoculum was approximately 10^7 per 10 μL. Control mussels were injected with 10 μL of the sterile phosphate buffer. Injected animals were kept in the Petri dishes overnight and 30 mL of ALW was added to the dish 12 hr after injection. Twenty of the treated mussels were incubated at room temperature (22±2°C) and observed for evidence of mortality. Death was confirmed by the opening of the shells and failure to respond to physical stimulation (21). All experiments were repeated at least three times independently and mean values were used in presenting the results.

Fractionation and concentration of bacterial metabolites

All glassware used in the following procedures was washed, rinsed with deionized water, and rinsed again with ultra-pure water (18Ω) before baking at 500°C overnight. Nutrient broth medium was used to culture each of the four selected isolates, HU-Z1, Z2, Z3, and Z4. Flasks containing the medium were incubated in a water bath shaker at 26°C. Bacteria in liquid culture at stationary phase were separated from the supernatant by centrifugation (10,000 × *g* for 20 min), and the supernatant was collected and aseptically filtered through a 0.2 μm-pore-size membrane filter (Gelman Sciences, Ann Arbor, MI, USA). The filtrate was concentrated using Filtron Mini-Ultrasette Tangential Flow Device (Filtron Technology Co., Northborough, MA, USA) to molecular-weight cut-offs of <5 kDa, 5-10 kDa and >10 kDa. The overall flow of fractionation procedures is presented schematically in Fig. 1. Concentrate fractions were lyophilized to dryness and used in subsequent tests of the effect of bacterial extracellular metabolites on zebra mussel mortality and analyses of chemical composition.

Methylene chloride extracts

Bacterial isolates, HU-Z2 and Z3, were selected and used to inoculate culture flasks containing nutrient broth and

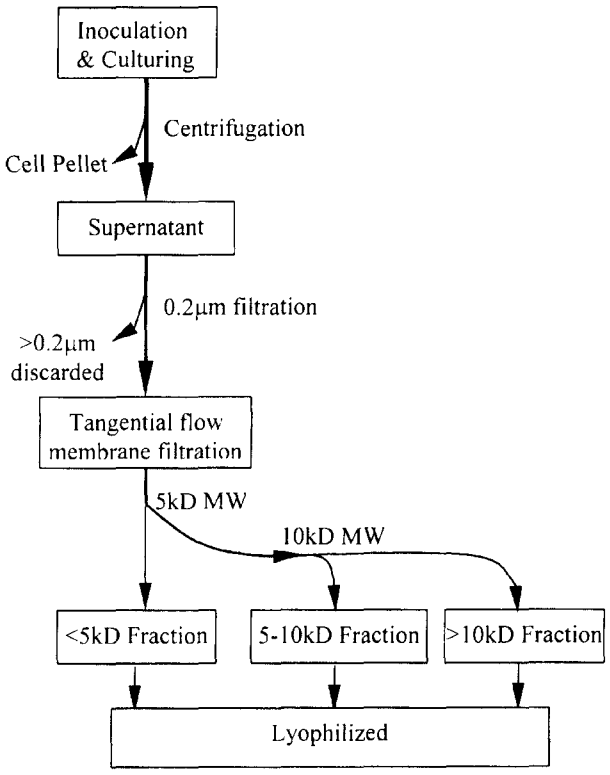

Fig. 1. A schematic diagram illustrating the fractionation procedures used in fractionating and concentrating extracellular metabolites based on molecular-weight cut-offs (<5, 5-10, and >10 kDa) from supernatants of bacterial isolates HU-Z1 (*Aeromonas media*), Z2 (*A. salmonicida*), Z3 (*A. veronii*), and Z4 (*Shewanella putrefaciens*).

incubated in a water bath shaker at 26°C. At the stationary phase, the culture suspension was centrifuged at 10,000 × *g* for 20 min. The supernatant was filtered through a 0.2 μm-pore-size filter (Gelman Sciences, Ann Arbor, MI, USA) and then extracted with 15 mL methylene chloride 3 times successively. The methylene chloride extracts were collected and evaporated in a rotory evaporator to a volume approximately 1 mL and then transferred to a conical tube which was under a positive pressure of pure nitrogen to complete dryness. The extract was redissolved in ultra-pure water before being used in the inoculation study on zebra mussels.

Inoculation of mussels

Similar to the bacterial inoculation method described above, groups of 20 mussels 9-15 mm in length were placed in sterile Petri dishes. These animals were conditioned to room temperature and then inoculated with 10 μL of bacterial metabolite concentrates in the abdominal side at a 30 degree angle from the rear. Injected animals were held in Petri dishes overnight and 30 mL of ALW was added to the dish 12 hr after injection. Treated mussels were incubated at room temperature for observation of mortality. Zebra mussel death was confirmed by the opening of their shells and failure to respond when they were touched with a sterile forceps (21). Experiments were repeated a minimum of three times.

Chemical analysis

Bacterial metabolites were analyzed for the presence of proteins, polysaccharide, DNA, 2-keto-3-deoxyoctonoic acid, and uronic acid. One mg of lyophilized material was dissolved in an appropriate volume of solvent. Five types of analysis were conducted: 1) proteins with Coomassie blue (3), 2) hexose sugars assayed by the anthrone method (5), 3) uronic acids by the *m*-phenylphenol method (2), 4) cell wall components by the thiobarbituric method for 2-KDO (32), and 5) deoxyribonucleic acid (DNA) by the diphenylamine method (28).

Results

Antagonism of bacterial isolates

Among the bacterial isolates obtained, four isolates were selected for further testing of their extracellular metabolites. The four isolates, HU-Z1, Z2, Z3 and Z4, were identified as *Aeromonas media*, *A. salmonicida*, *A. veronii*, and *Shewanella putrefaciens*, respectively. The antagonism of bacterial isolates to adult mussels was evaluated by inoculating individual animals with mid-log phase cell concentrates. At a dosage of 10^7 cells per mussel, mortality reached 100% after 5 days of incubation with *A. media*, *A. veronii*, and *S. putrefaciens*, but only 61% with *A. salmonicida* (Fig. 2). Patterns of the progressive increase

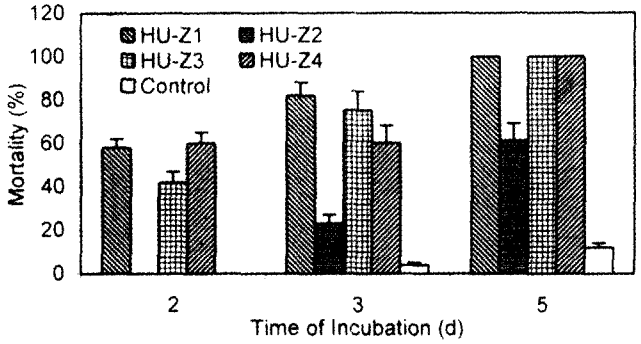

Fig. 2. Antagonistic effects of bacterial isolates HU-Z1 (*A. media*), Z2 (*A. salmonicida*), Z3 (*A. veronii*), and Z4 (*S. putrefaciens*) on zebra mussels during incubation at 22°C. Inoculation dose of bacterial suspension was 10^7 cells per mussel. Data presented are means (± standard deviation, vertical line) of three independent tests.

of mussel mortality were almost identical for isolates HU-Z1 and Z3. During the same time period, the control mussel group, receiving phosphate buffer solution, resulted in 0 to 12% mortality in all tests.

Antagonism of extracellular metabolites from bacteria

Crude extracellular metabolites concentrated from the culture supernatant of the bacterial isolates, HU-Z1, Z2, Z3, and Z4, displayed antagonistic effects on mussels after inoculation. Among the three fractions (<5 kDa, 5-10 kDa, and >10 kDa) obtained in this study, fractions of higher molecular weight resulted in higher mortality within 2 days of incubation (Fig. 3). Results of 100% mortality were observed with >10 kDa fraction of isolates Z2, Z3 and Z4 within two days of inoculation. Similarly, 5-10 kDa fraction showed better performance than <5 kDa fraction for HU-Z2, Z3 and Z4. However, the intermediate 5-10 kDa fraction of isolate HU-Z1 seemed to be more effective than the other two fractions.

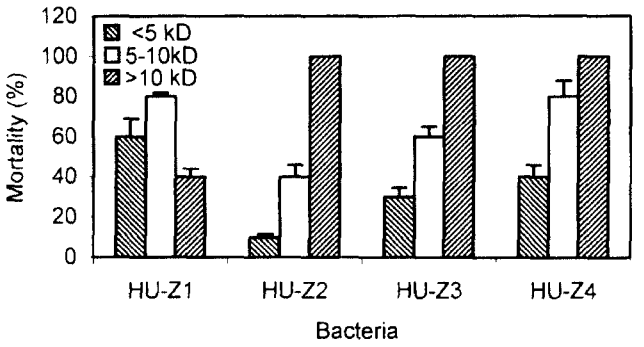

Fig. 3. Antagonistic effects of extracellular metabolites concentrated based on molecular-weight cut-offs (<5, 5-10, and >10 kDalton) from supernatants of bacterial isolates HU-Z1 (*A. media*), Z2 (*A. salmonicida*), Z3 (*A. veronii*), and Z4 (*S. putrefaciens*) on zebra mussels at 22°C within 2 days of incubation. Data presented are means (± standard deviation, vertical line) of three independent tests.

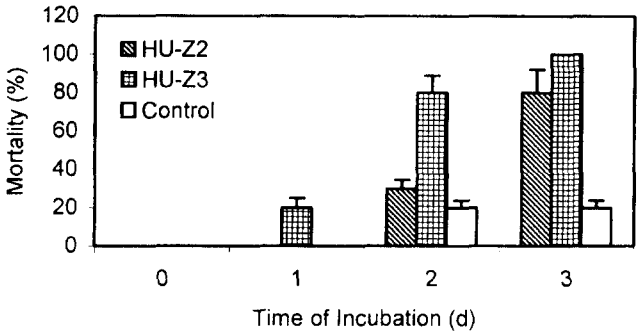

Fig. 4. The antagonistic effects of methylene chloride extracts of bacterial isolates Z2 (*A. salmonicida*) and Z3 (*A. veronii*) supernatants on mussel mortality. Data presented are means (± standard deviation, vertical line) of three independent tests.

Furthermore, methylene chloride-extracted extracellular metabolites of Z2 and Z3 were also assayed for their antagonism to zebra mussels. Extracts of both bacterial isolates were strongly antagonistic to the mussels and as high as 80% mortality was observed with HU-Z3 within 2 days of inoculation. The difference between the two isolates in mussel mortality became narrower as the length of incubation time was extended (Fig. 4). A mortality of 100% was reached with Z3 extract within 3 days while only 80% mortality was observed with Z2 during the same time period.

Chemical analysis of the fractionated materials

Chemical characterization of the 5-10 kDa and the >10 kDa fractions indicated the presence of polysaccharides and proteins (Table 1), but there was no detectable presence of 2-keto-3-deoxyoctanoic acid (2-KDO), uronic acid, or deoxyribunucleic acids (DNA). Proteins accounted for 3.2-4.2% of the lyophilized crude material for the 5-10 kDa fraction, whereas they were between 1.7 and 4.9% in the >10 kDa fraction. However, the polysaccharide concentration was much higher and more variable than the proteins, 9.3-22% for the 5-10 kDa fraction and 5.2-57% for the >10 kDa fraction. Significant quantities of the lyophilized material could not be identified.

Table 1. Chemical characterization of extracellular metabolites (5-10 kDa and >10 kD) concentrated from supernatants of bacterial isolates HU-Z1 (*A. media*) and Z2 (*A. salmonicida*), Z3 (*A. veronii*) and Z4 (*S. putrefaciens*)

Isolate	5-10 kDa		>10 kDa	
	Polysaccharides	Protein	Polysaccharides	Protein
	($\mu g\ mg^{-1}$ lypholized materials)			
HU-Z1	40.7 ± 1.8	34.9 ± 1.6	51.5 ± 1.9	26.9 ± 1.8
HU-Z2	200.4 ± 15.9	32.2 ± 3.2	273.4 ± 21.2	17.0 ± 2.0
HU-Z3	223.4 ± 26.0	40.4 ± 3.2	571.3 ± 18.5	24.8 ± 0.6
HU-Z4	93.2 ± 6.5	42.2 ± 5.0	108.9 ± 3.2	49.2 ± 1.7

Discussion

Bacteria are involved in pathogenicity and disease in animals, particularly in aquatic species. In this study, we demonstrated that both bacterial isolates and their extracellular metabolites exhibited antagonistic effects against the zebra mussel, *Dreissena polymorpha*. The lethality was dependent on individual isolates and the effectiveness of the extracellular metabolites was dependent on molecular size fractions.

Extracellular metabolites of marine bacterium D2 have been reported to be lethal to the barnacle larvae, *Balanus amphitrite* (18). The bacterium was isolated from the surface of an adult tunicate. Subsequent 16S rRNA sequencing revealed its identity as a member of the γ-subgroup of *Proteobacter*. A low molecular weight molecule (<500 Da) product from the bacterium has been isolated but showed no evidence of protein or peptide moieties, while a high molecular weight substance inhibits and prevents the settlement of barnacle larvae, as well as inhibiting a range of marine bacterial species (18).

Gram negative bacteria are known for their production of lipopolysaccharide (LPS) or endotoxins. LPS is a major constituent of the outer membrane of Gram-negative bacteria, and its terminal disaccharide phospholipid (Lipid A) portion contains the key structural features responsible for toxic activity (30). Bacterial metabolites include a wide range of chemicals which vary among species (29, 31, 36). Ford *et al.* reported a predominance of polysaccharides and proteins in bacterial exopolymers isolated from aquatic environments and uronic acid was detected in four of the 11 isolates tested (8). The four isolates used in this study were *A. media*, *A. salmonicida*, *A. veronii*, and *S. putrefaciens*. However, no uronic acid was detected in any of the isolates in the current study. On the basis of methylene chloride extraction and polarity of the solvent, the active component is likely polar molecules.

Many marine bacteria have been found to produce antibiotics and brominated compounds which may prevent fouling and assist in the competition for space and food (35). Natural products, alkaloids or bryostatins isolated from bryozoans, are known to be both antifoulants and antibacterial agents (1). Aged films of the bacterirum *Deleya marina* are more inhibitory to the attachment of the larvae *B. amphitrite* than freshly formed films (22, 23, 25). These researchers speculated that the bacterial extracellular materials may be involved in the inhibitory process.

Both bacteria and their metabolites are known to affect the attachment of marine invertebrates. Settlement of larvae can be either stimulated or inhibited by biofilms of bacteria on surfaces (11, 17, 18, 22-26). Indigenous bacteria can be opportunistic pathogens killing mussels and the lethality involves the extracellular metabolites of high-molecular weight produced by the bacteria.

In conclusion, we have demonstrated that both bacterial isolates and extracellular metabolites of the bacteria are antagonists to zebra mussels. The most effective fraction was >10 kDa in molecular weight and the active components are polar molecules. This study showed that, in addition to bacterial pathogenicity, extracellular metabolites are also involved in the antagonism between bacteria and host. Further elucidation of the chemical structure of the active metabolite and the mechanisms will further our strategies in anti-fouling control.

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