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Effects of symbiotic bacteria on chemical sensitivity of *Daphnia magna*

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ABSTRACT

The crustacean zooplankton *Daphnia magna* has been widely used for chemical toxicity tests. Although abiotic factors have been well documented in ecotoxicological test protocols, biotic factors that may affect the sensitivity to chemical compounds remain limited. Recently, we identified symbiotic bacteria that are critical for the growth and reproduction of *D. magna*. The presence of symbiotic bacteria on *Daphnia* raised the question as to whether these bacteria have a positive or negative effect on toxicity tests. In order to evaluate the effects of symbiotic bacteria on toxicity tests, bacteria-free *Daphnia* were prepared, and their chemical sensitivities were compared with that of *Daphnia* with symbiotic bacteria based on an acute immobilization test. The *Daphnia* with symbiotic bacteria showed higher chemical resistance to nonylphenol, fenoxycarb, and pentachlorophenol than bacteria-free *Daphnia*. These results suggested potential roles of symbiotic bacteria in the chemical resistance of its host *Daphnia*.

1. Introduction

The zooplankton *Daphnia magna* is a small crustacean, which inhabits various aquatic environments and plays an important role in ecosystems by having a pivotal role in the food web. In addition to their ecological importance, *Daphnia* have long been used as a model organism for toxicity testing in the laboratory because they are highly sensitive to environmental changes, have a short life cycle, are small in size, and are easy to handle, culture, and reproduce. They are used as a model for toxicity tests, standardized in accordance with OECD (Organization for Economic Co-operation and Development) test guidelines.

In toxicity tests, the effects of chemicals on *Daphnia* are evaluated by observing changes in growth, reproduction, swimming behavior, and other traits (Persoone et al., 2009). In one of the standard tests, assigned by the OECD, immobilization, and male production and reproduction are evaluated after chemical exposure (OECD, 2004), (OECD, 2012). In these standardized toxicity tests, abiotic factors such as pH, hardness of medium, temperature, and photoperiod have all been well documented in the test protocols because it is well known that these factors influence the life-history traits of daphniids.

On the other hand, biotic factors are not well documented in the test protocols. Recently, it has been reported that symbiosis is one of the biotic factors that affect the life history traits of many species. Studies on the effects of symbiotic bacteria in a wide variety of host species, including humans have been conducted (Kikuchi et al., 2012). The term “symbiosis” implies an interaction between organisms, which can be categorized in three groups: parasitism, commensalism, and mutualism (Wells and Varel, 2011). Parasitism is a relationship, in which the parasite organism benefits to the detriment of the host. This interaction has been widely studied in humans, animals, and plants to investigate methods of preventing widespread diseases (Wells and Varel, 2011). In *Daphnia*, several studies have revealed parasitic interactions between *Daphnia* and parasites. These parasites control *Daphnia's* life history traits and function in the host-parasite coevolution (Decaestecker et al., 2007), (Auld et al., 2012), (Ebert, 2008). Commensalism generally refers to two organisms living together, without an associated cost but without an obvious benefit to either organism (Hooper and Gordon, 2001). Mutualism is a beneficial interaction where two organisms interact and both benefit (Wells and Varel, 2011). In

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addition to parasitism studies, the effects of biotic factors have been studied in terms of stress of predators (Cousyn et al., 2001). Interactions between chemicals and biotic factors have also been studied (Bernatowicz and Pijanowska, 2011), (Hochmuth et al., 2016), (Jansen et al., 2011) (Shurin and Dodson, 1997),. However, studies on the effect of symbiotic bacteria, one of the biotic factors, and chemical stress remain limited (Gorokhova et al., 2015).

Bacteria-free *Daphnia* have been prepared in previous studies, which comparatively evaluated biological traits such as growth and reproduction (Peerakietkhajorn et al., 2015) (Sison-Mangus et al., 2015), with *Daphnia* containing symbiotic bacteria (hereafter, symbiotic *Daphnia*). In the present study, we found that some symbiotic bacteria have positive effects on *Daphnia*. Regarding reproduction, normal *Daphnia* gave larger population size than bacteria-free *Daphnia*. Inoculation of bacteria-free *Daphnia* with a strain of *Limnohabitans* sp. a symbiotic bacterium, restored the host fecundity to the same level as the symbiotic *Daphnia* (Peerakietkhajorn et al., 2016). Recently, metagenomics of symbionts in *D. pulex*, *D. pulicaria,* and *D. magna* have been investigated using shotgun sequencing. This study revealed that the bacterial community compositions are stable among these three species and the majority of the microbial community is Proteobacteria. Most sequences belong to the Betaproteobacteria family Comamonadaceae (Qi et al., 2009). Our study also showed that *Limnohabitans* sp. Belonging to Betaproteobacteria play an essential role in conferring fecundity by showing that bacteria-free *Daphnia* recovers fecundity when inoculated with *Limnohabitans* sp. (Peerakietkhajorn et al., 2016). Another study also found that *Limnohabitans* sp. can be found on the filter of *Daphnia*, where dissolved organic carbon is efficiently supplied (Eckert and Pernthaler, 2014). Enrichment of *Limnohabitans* sp. on *Daphnia*, rather than environmental water, suggests that the environment of *Daphnia*, including dissolved organic carbon, is desirable to *Limnohabitans* sp. The stable existence of *Limnohabitans* sp. on *Daphnia* suggests that these symbiotic bacteria are stably transmitted (Freese and Schink, 2011). These finding indicate that some bacteria co-exist in a mutualistic relationship in *Daphnia*.

This finding prompted us to examine the effects of symbiotic bacteria on toxicity tests, because it might be possible that symbiotic bacteria enhance chemical resistance as well as fitness to the environment (Peerakietkhajorn et al., 2015). In this study, the objective was to examine the effect of symbiotic bacteria on the sensitivity of *Daphnia magna* to anthropogenic chemicals. For this purpose, we prepared bacteria-free (sterilized) *Daphnia* and performed acute immobilization tests. We also carried out these tests on *Daphnia* with normal symbiotic microbial flora. As hypothesized, the results of the acute immobilization test revealed that normal *Daphnia* were more resistant to nonylphenol, fenoxycarb, and pentachlorophenol than bacteria-free *Daphnia*.

2. Material and methods

2.1. Chemicals

Nonylphenol, fenoxycarb, pentachlorophenol, and triclosan were purchased from Nacalai Tesque (Kyoto, Japan) and all chemicals were dissolved in dimethylformamide.

2.2. Daphnia strain and cultivation

Daphnia magna (NIES strain) were obtained from the National Institute for Environmental Studies, Japan (Oda, 2006). Eighty daphniids were cultured in 5 L ADaM (Artificial Daphnia Medium) (Kluttgen et al., 1994) at 24 ± 1 °C and a 16-h light/8-h dark photoperiod. Subsequently, 2×10^9 cells of *Chlorella sp*. were added daily in the first week, and then 4×10^9 cells daily thereafter. This daphnia stock was used to obtain embryos.

2.3. Determination of the optimal Daphnia embryonic stage required for the preparation of bacteria-free Daphnia

In order to compare sensitivity to chemicals between bacteria-free and normal *Daphnia*, we determined the optimal period suitable for the elimination of bacteria. Adult daphniids were transferred to M4 culture medium filtrated with a 0.2-μm filter and the embryos were isolated from the brood chambers. Embryos at 0, 6, 12, and 18 h after ovulation were used for glutaraldehyde treatment. The isolated embryos were exposed to different concentrations of glutaraldehyde (Sigma-Aldrich, St. Louis, MO) (0.0025%, 0.025%, 0.25%, and 2.5%) for 30 min and washed twice with the filtered M4 medium. They were then incubated in 4 mL of the filtered M4 medium in six well plates at 23 °C and maintained under a 16-h light/8-h dark photoperiod. The survival rate (%) of daphniids after hatching from the vitellin membrane (ca. 72 h after ovulation) was determined and the copy number of bacterial 16S rDNA/daphniids was evaluated by quantitative PCR (qPCR).

2.4. Preparation of bacteria-free Daphnia

Based on the optimized time points, bacteria-free *Daphnia* were prepared. In order to prepare bacteria-free *Daphnia*, adult daphniids with appropriate stage embryos were transferred to M4 culture medium (Elendt and Bias, 1990) and filtrated with a 0.2-μm filter (Milex-LG 0.2 μm, Millipore, Billerica, MA). The embryos at the 12 and 18 h developmental stages were isolated from brood chambers and exposed to 0.25% glutaraldehyde for 30 min, washed twice with the filtered M4 medium, and then incubated in 4 mL of the filtered M4 medium of six well plates at 23 °C.

As a control, embryos at the 12 h and 18 h developmental stages were dissected, washed twice with filtered M4 medium, and then incubated in 4 mL of the filtered M4 medium in six well plates at 23 °C. As these embryos were omitted from the glutaraldehyde treatment step, symbiotic bacteria remained on the *Daphnia*, as confirmed by qPCR analysis of 16S rDNA.

2.5. Quantification of bacterial DNA using qPCR of 16S rDNA

To evaluate the presence of bacteria on *Daphnia*, total DNA was prepared from *Daphnia* and co-purified bacterial DNA, and was quantified by qPCR. Ten daphniids were homogenized in 200 μL Buffer A (100 mM Tris-HCl at pH 7.5, 100 mM ethylenediaminetetraacetic acid, 100 mM NaCl and 0.5% sodium dodecyl sulfate) with zirconia beads (φ1.0 and φ3.0 (Tomy, Tokyo, Japan)). Homogenization was carried out using Micro Smash MS-100 (Tomy, Tokyo, Japan) for 30 s at 3000 rpm four times followed by incubation at 65 °C for 30 min. Four hundred microliters of KAc/LiCl solution (5 M potassium acetate:6 M lithium chloride = 1:2.5) was added and the solution was incubated on ice for 10 min, and subsequently centrifuged at 15,000 rpm for 10 min. The resulting supernatant was mixed with isopropanol, precipitated, and used for quantitative PCR (qPCR).

qPCR was performed by MX3005P (Stratagene) using SYBR GreenER qPCR SuperMix Universal (Invitrogen), in the presence of forward primer 5′-AGACACGGTCCAGACTCCTAC-3′ and reverse primer 5′-TTTACGGCGTGGACTACCAG-3′. The primers were the same as those used for the quantification of 16S rDNA in a previous study (Peerakietkhajorn et al., 2015). PCR amplifications were performed under the following conditions: 2 min at 95 °C followed by forty two-temperature cycles of 15 s at 95 °C and 1 min at 60 °C.