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Effect of Heat Sterilization on the Bioactivity of Antibacterial Metabolites Secreted by *Xenorhabdus nematophila*

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Abstract: *Photorhabdus luminescens* and *Xenorhabdus nematophila* are entomopathogenic bacterial symbionts of beneficial nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*, respectively. These bacterial symbionts are known to secrete an array of toxins, enzymes and antimicrobials that kill, bioconvert and protect the insect host for optimal nematode growth and reproduction. The present study explores heat stability of antibacterial metabolites secreted by *X. nematophila*. Permeate of a liquid *X. nematophila* culture was subjected to various sterilization treatments to observe the effects of heat sterilization on antibacterial activity. Activity was measured as bacterial sensitivity which is assayed utilizing a modified-version of the Kirby-Bauer disk diffusion method. Results demonstrate that *X. nematophila* produces both heat-labile and heat-stabile antibacterials that are effective against different species of bacteria. Results also indicated that heat-stabile components are more active than heat-labile components. The discovery of an environmental organism that produces both heat-stabile and heat-labile antibacterials can be exploited to manufacture these compounds for potential medical applications for human and animal use.

Key words: *Xenorhabdus nematophila*, antibacterials, sterilization, *Steinernema carpocapsae*, *Photorhabdus luminescens*

INTRODUCTION

Xenorhabdus nematophila is a species of Gram-negative, entomopathogenic bacteria that symbiotically associates with insect-parasitizing nematodes of *Steinernema carpocapsae*. As *S. carpocapsae* nematodes attack an insect host, they navigate to the insect hemocoel where they regurgitate *X. nematophila* from their intestinal tract into the insect hemolymph (Boemare *et al*., 1996). As the bacterial symbiont proliferates within the hemolymph, the symbiont secretes an array of insecticidal toxins and degradating enzymes that kill and bioconvert the insect into dietary components for both symbiotic partners. This symbiotic and pathogenic relationship has also been observed in other nematode-bacterial complexes (Forst and Nealson, 1996). *X. nematophila*, along with other species belonging to the genus, secretes antibacterial metabolites into the insect hemolymph. Furthermore, these metabolites have been strongly speculated to prevent putrification of the insect cadaver by competing bacteria and as a result, ideal conditions for nematode growth and reproduction are produced (El-Hag and El-Sadawy, 2008; Singh *et al*., 2012; Inman *et al*., 2012; Paul *et al*., 1981).

In a recent study, researchers concluded that antibacterials produced by *Photorhabdus luminescens* (bacterial symbiont of *Heterorhabditis bacteriophora* nematodes) are secreted and active in liquid culture media (Inman and Holmes, 2012). They also showed that filter-sterilized metabolites were effective against numerous bacterial species; however, they did utilize heat-sterilization. In this communication, the study demonstrates the effects of sterilization techniques on the bioactivity of antibacterial compounds secreted by *X. nematophila*. Determination of metabolite bioactivity for both sterilization treatments along with a non-sterilized sample was performed utilizing the Kirby-Bauer disk diffusion antibiotic susceptibility method (Bauer *et al*., 1966) that was modified in a previous study (Inman and Holmes, 2012).

MATERIALS AND METHODS

Xenorhabdus nematophila (ATCC 39497) was employed in this study. Upon isolation on Brain Heart Infusion (BHI) agar, colonies of *X. nematophila* were upscaled in BHI broth for reactor inoculation. A Sartorius stedim Biostat® A-plus bioreactor containing BHI was

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inoculated with fresh cells from an overnight culture of the bacterial symbiont. Process parameters that were held constant include: agitation-300 rotations per minute (rpm); aeration-1 volume of air to 1 volume of liquid medium per minute (vvm); pH (7.30); temperature (28°C); and dissolved oxygen percent-30%. The process was ended one hour into the stationary phase of growth (~12 h) as monitored by a cell density probe. The bacterial culture was then subjected to cross-flow filtration utilizing a 50 kDa cutoff filter cassette to generate a cell-free permeate. The obtained permeate was divided into three aliquots and treated as follows: 100 mL non-sterilized, 100 mL filter-sterilized and 100 mL heat sterilized (autoclaved). Sterile, blank disks were divided into four groups and impregnated with: sterile BHI broth; non-sterilized; filter-sterilized and heat-sterilized.

Colonies of each screening microbe were used to inoculate 5 mL culture tubes containing BHI. Culture tubes were incubated at the microbes' respective optimal temperature until the culture turbidity was comparable to a 0.5 MacFarland standard. Culture aliquots of 0.1 mL of each microbe were spread onto Muller-Hinton plates to produce lawns of bacterial growth. Impregnated discs were placed onto the freshly inoculated agar surface and incubated overnight respectively to optimal temperatures of the screening organisms. Each microbe was screened thrice and the diameter of each zone was averaged.

RESULTS

Bioactivity (i.e., zones of sensitivity) of the metabolites exposed to various sterilization treatments is depicted in Table 1. Results indicate that *X. nematophila* does secrete antibacterials into the culture medium. Additionally, it is observed that these antibacterials consist of both heat-labile and heat-stabile compounds.

For eight of the screened microbes, heat sterilized components account for the majority of the bioactivity seen when compared to the non- and filter-sterilized permeates. However, the increased zones seen with non- and filter-sterilized permeates is speculated to be caused by the synchronous activity of both heat-labile and heat-stabile components. For these cases, both components had inhibitory effects and seem to work in conjunction with each other as seen in the case with *Bacillus cereus* (Table 1). The heat active component resulted in a zone diameter of 7 mm; however, both non- and filter-sterilized permeate exhibited an inhibitory zone of 10 mm. Due to the presence of both heat-stabile and heat-labile components in both non- and filter-sterilized permeates, the inhibitory zone that is caused by heat-labile components can be calculated from the subtraction of the zone of activity caused by the heat-stabile component (7 mm) from the inhibitory zone of the combine components (10 mm). This results in a zone diameter of 3 mm for the heat labile components.

Other findings include 4 cases (*Escherichia coli*, *Neisseria gonorrheae*, *N. sicca* and *Staphylococcus aureus*) which the microorganisms tested were not affected by heat-stabile components (0 mm); however, they were only sensitive to the heat-labile components found within non- and filter sterilized permeates which exhibited zone sizes of 11, 8, 7 and 10 mm, respectively. Furthermore, two species (*Bacillus megaterium* and *Neisseria subflava*) exhibited zones that were identical in diameter regardless of treatment with diameters of 11 mm and 8 mm, respectively. This finding suggests that these two microbes were only sensitive to the heat-stabile

Table 1: Zones of sensitivity for each bacterial species and sterilization technique utilized

	Zone diameter (mm)			
Organism	Non-sterilized	Filter-sterilized	Heat-stabile	Heat-labile[c]
Bacillus cereus	10	10	7	3
B. coagulans	9	9	7	2
B. licheniformis	8	8	7	1
B. megaterium[a]	11	11	11	0
B. subtilis	10	10	8	2
Escherichia coli[b]	11	11	0	11
Micrococcus luteus	13	13	11	2
Moraxella catarrhalis	9	9	8	1
Neisseria gonorrheae[b]	8	8	0	8
N. sicca[b]	7	7	0	7
N. subflava[a]	8	8	8	0
Staphylococcus aureus[b]	10	10	0	10
S. saprophyticus	13	13	8	5
S. mutans	10	10	8	2

[a]:Affected by heat-stabile only, [b]:Affected by heat-labile only, [c]:Calculated by subtracting heat-stabile from filter-sterilized permeate

component(s). Organisms tested that were not sensitive were excluded from the analysis. According to the presented data, both non-sterilized and filter-sterilized permeates contain all active compounds, both heat-labile and heat-stabile; however, heat-stabile metabolites were only detected in the heat-sterilized permeate.

DISCUSSION

The results of the present study demonstrate that *Xenorhabdus nematophila* produces both heat-stabile and heat-labile antibacterials that are bioactive against other bacteria. Since *X. nematophila* secretes these metabolites into the culturing medium, it can be speculated that these compounds are secreted into the insect hemolymph where they work simultaneously with each other to decrease the chance of putrification by competing microbes. Several reports suggest that *Xenorhabdus* spp. produce bioactive compounds such as xenocoumacins, indoles and dithiolopyrrolones (McInerney *et al*., 1991a, b; Sundar and Chang, 1993); however, they do not report on the thermal stability of these compounds. Furthermore, El-Hag and El-Sadawy (2008) demonstrated the existence of an antimicrobial peptide agent that greatly affected *Staphylococcus aureus*. Based upon the data obtained for *S. aureus* from this study, it is likely that the heat-labile component found within the non- and filter-sterilized permeate is the previously reported antibacterial protein. Antibacterial proteins are considered to be potent as they can be utilized as broad-spectrum antibacterial therapeutics. As far as the results are concerned for the heat-sterilized permeate where no bioactivity was seen, it is concluded that the antibacterial protein was thermally denatured therefore hindering its effectiveness against *S. aureus*. In all of the sensitive microbes screened (Table 1), all but six were affected by a combination of both components; however, the remaining six were only affected by either heat-stabile components (*B. megaterium* and *N. subflava*) or heat-labile components only (*E. coli*, *N. gonorrheae*, *N. sicca* and *S. aureus*).

CONCLUSION

The preliminary discovery of secreted heat stabile components that show antibacterial activity is novel as most, if not all, currently used antibiotics are sensitive to thermal stresses (Hsieh *et al*., 2011). The data obtained from this study supports the findings of antibacterial proteins secreted by the *Xenorhabdus* spp. The identity of these compounds is currently unknown to the authors and as a consequence additional research is necessary to isolate, identify and characterize these molecules. By determining their physicochemical and antibacterial properties, both heat-stabile and heat-labile bacterial metabolites/proteins may prove to be useful in treating bacterial diseases of both humans and animals.

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