VIRULENCE MECHANISMS OF THE NEMATODE PHASMARHABDITIS HERMAPHRODITA AND ITS ASSOCIATED BACTERIUM MORAXELLA OSLOENSIS TO THE GRAY GARDEN SLUG DEROCERAS RETICULATUM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Li Tan, M.S.

* * * * *

The Ohio State University

2002

Dissertation Committee:                                    Approved by
Dr. Parwinder S. Grewal, Adviser
Dr. David L. Denlinger
Dr. Donald H. Dean
Dr. Ronald B. Hammond

Department of Entomology
ABSTRACT

_Moraxella osloensis_, a gram-negative bacterium, is associated with _Phasmarhabditis hermaphrodita_, a lethal slug-parasitic nematode that has potential for the biocontrol of mollusk pests, especially the gray garden slug _Deroceras reticulatum_. We discovered that the shell cavity in the posterior mantle region of _D. reticulatum_ served as the main portal of entry for _P. hermaphrodita_. The nematode is a facultative parasite of the slug and only dauer stage can serve as an infective stage in the natural environment. Aged _M. osloensis_ cultures were pathogenic to _D. reticulatum_ after injection into the shell cavity or hemocoel of the slug. _P. hermaphrodita_ vectors _M. osloensis_ into the shell cavity and the bacterium is the main killing agent in the nematode/bacterium complex. We also discovered that _M. osloensis_ lipopolysaccharide (LPS) was an endotoxin that was active against the slug. Purified _M. osloensis_ LPS had a lethal injection toxicity but no contact or oral toxicity against the slug. Toxicity of _M. osloensis_ LPS resides in the lipid A moiety but not in the polysaccharide moiety. The LPS was a rough-type LPS with an estimated molecular weight of 5,300. Coinjection of galactosamine with the LPS increased its toxicity to _D. reticulatum_ by 2-4 fold. The galactosamine-induced sensitization was reversed completely by uridine. We further discovered that 1 or 2-day _M. osloensis_ cultures were non or less pathogenic whereas 3 to 5-day _M. osloensis_ cultures were more pathogenic to the slug. The average yield of _M. osloensis_ LPS per bacterium did not differ among the 1 to 5-day cultures. However, _M.
*osloensis* cells from the 3-day cultures produced more outer membrane proteins than those from the younger or older cultures. The intensity and pattern of *M. osloensis* aggregation changed with time of culture. Pili-like projections were rarely present on the bacterial surfaces of *M. osloensis* from 1-day cultures, but reached maximal density in 3-day cultures. The temporal expression of the pili-like projections strongly correlates with the temporal pattern of *M. osloensis* virulence to *D. reticulatum*. The changes of *M. osloensis* pathogenicity against *D. reticulatum* during culture strongly correlate with structural changes in the bacterial cell wall.
Dedicated to my parents and wife
ACKNOWLEDGMENTS

I thank sincerely my advisor, Dr. Parwinder S. Grewal, for his intellectual guidance throughout my graduate program. His great support, encouragement, enthusiasm, and patience made this dissertation become possible. What I have learned from him has prepared me profoundly for future opportunities.

I also appreciate other members of my advisory/dissertation committee, Dr. David L. Denlinger, Dr. Donald H. Dean, and Dr. Ronald B. Hammond for their invaluable guidances on my experimental designs and precious comments on my dissertation. Help with slug biology and taxonomy offered by Dr. Hammond was very valuable. Furthermore, recommendations on my application for the presidential fellowship from Dr. Grewal, Dr. Denlinger, Dr. Dean, Dr. Larry Phelan, and Dr. Steve Forst (University of Wisconsin, Milwaukee) are greatly appreciated.

I am also grateful to MicroBio Ltd (Cambridge, U.K.) for providing the nematode and its associated bacterium used in my experiments, to Dr. Tea Meulia, Ms. Karli J. Fitzelle, and Mr. Dave Fulton for proving technical support on electron microscopy, to Ms. Judy A. Smith for collecting slugs for my experiments, and to all of my lab colleagues for their care, help, and friendship, and for creating a very pleasant working environment.
environment for me, especially Dr. Sukhbir Grewal, Dr. Ganpati Jagdale, Dr. Seppo Salminen, Dr. Douglas Richmond, Dr. Elizabeth De Nardo, Mr. Kevin Power, Mr. Brian Kunkel, and Ms. Corrie Yoder.

I would like to specially express my deepest appreciation to my parents and wife in China. Without their love and support, I could not go so far.

This work was supported by a matching fund grant from the Ohio Agricultural Research and Development Center (OARDC) and MicroBio Ltd, Cambridge, U.K. to Dr. Parwinder S. Grewal, by an OARDC Graduate Research Competitive Grant and a Presidential Fellowship from the Ohio State University to Li Tan.
VITA

Oct. 1, 1973..............................Born - Youxian, Hunan Province, P. R. China

1991-1995...............................B.S. Microbiology, Shandong University, 
                                 Jinan, Shandong Province, P. R. China

1995-1998...............................M.S. Entomology, Shanghai Institute of Entomology, 
                                 Chinese Academy of Science, Shanghai, P. R. China

1998-2001...............................Graduate Research and Teaching Associate, 
                                 The Ohio State University

2002.................................Presidental Fellow, The Ohio State University

PUBLICATIONS

Refereed Publications


Tan, L., and P. S. Grewal. 2002. Endotoxin activity of Moraxella osloensis against the 

Tan, L., and P. S. Grewal. 2001. Pathogenicity of Moraxella osloensis, a bacterium 
 associated with the nematode Phasmarhabditis hermaphrodita, to the grey garden 

Abstracts


Proceeding


FIELDS OF STUDY

Major Field: Entomology

Study in Nematology and Invertebrate Microbiology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td></td>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td></td>
<td><strong>Chapters:</strong></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Infection behavior of the rhabditid nematode <em>Phasmarhabditis hermaphrodita</em> to the gray garden slug <em>Deroceras reticulatum</em></td>
<td>14</td>
</tr>
<tr>
<td>2.1</td>
<td>Abstract</td>
<td>14</td>
</tr>
<tr>
<td>2.2</td>
<td>Introduction</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Materials and Methods</td>
<td>16</td>
</tr>
<tr>
<td>2.4</td>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>2.5</td>
<td>Discussion</td>
<td>23</td>
</tr>
<tr>
<td>2.6</td>
<td>Acknowledgments</td>
<td>25</td>
</tr>
<tr>
<td>2.7</td>
<td>References</td>
<td>26</td>
</tr>
<tr>
<td>3.</td>
<td>Pathogenicity of <em>Moraxella osloensis</em>, a bacterium associated with the nematode <em>Phasmarhabditis hermaphrodita</em>, to the slug <em>Deroceras reticulatum</em></td>
<td>33</td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>33</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>34</td>
</tr>
<tr>
<td>3.3</td>
<td>Materials and Methods</td>
<td>37</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>42</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td>3.6</td>
<td>Acknowledgments</td>
<td>48</td>
</tr>
<tr>
<td>3.7</td>
<td>References</td>
<td>48</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Host range of <em>P. hermaphrodita</em></td>
<td>3</td>
</tr>
<tr>
<td>1.2 Bacteria associated with <em>P. hermaphrodita</em></td>
<td>5</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic diagram of <em>D. reticulatum</em> dissections</td>
<td>18</td>
</tr>
<tr>
<td>2.2</td>
<td>Time and route of invasion of <em>P. hermaphrodita</em> into <em>D. reticulatum</em></td>
<td>28</td>
</tr>
<tr>
<td>2.3</td>
<td>Dauer recovery of <em>P. hermaphrodita</em> in non-parasitic conditions</td>
<td>29</td>
</tr>
<tr>
<td>2.4</td>
<td>Multiplication of <em>P. hermaphrodita</em> in non-parasitic conditions</td>
<td>30</td>
</tr>
<tr>
<td>2.5</td>
<td>Infectivity of different life stages of <em>P. hermaphrodita</em> to <em>D. reticulatum</em> at 15 days after external exposure to the slugs</td>
<td>31</td>
</tr>
<tr>
<td>2.6</td>
<td>Infectivity of different life stage of <em>P. hermaphrodita</em> to <em>D. reticulatum</em> at 15 days after injection into the shell cavity</td>
<td>32</td>
</tr>
<tr>
<td>3.1</td>
<td>Percentage mortality (mean ± SE, n = 3) of <em>D. reticulatum</em> following injection of different concentrations (CFU/slug) of 40-hr <em>M. osloensis</em> culture into the shell cavity</td>
<td>52</td>
</tr>
<tr>
<td>3.2</td>
<td>Percentage mortality (mean ± SE, n = 3) of <em>D. reticulatum</em> following injection of different concentrations (CFU/slug) of 60-hr <em>M. osloensis</em> culture into the shell cavity</td>
<td>53</td>
</tr>
<tr>
<td>3.3</td>
<td>Percentage mortality (mean ± SE, n = 3) of <em>D. reticulatum</em> following injection of <em>M. osloensis</em> (60-hr culture) with or without the antibiotics into the shell cavity</td>
<td>54</td>
</tr>
<tr>
<td>3.4</td>
<td>Percentage mortality (mean ± SE, n = 3) of <em>D. reticulatum</em> following injection of cultures of <em>M. osloensis</em> of different ages of into the hemocoel</td>
<td>55</td>
</tr>
<tr>
<td>3.5</td>
<td>Percentage of infective juveniles of <em>P. hermaphrodita</em> from a fresh, a 3-month old, and an 8-month old batch in different groups</td>
<td>56</td>
</tr>
<tr>
<td>3.6</td>
<td>Percentage mortality (mean ± SE, n = 3) of <em>D. reticulatum</em> following injection of axenic and aged <em>P. hermaphrodita</em> into the shell cavity</td>
<td>57</td>
</tr>
</tbody>
</table>
4.1 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of different components of 3-day *M. osloensis* cultures into the shell cavity……76

4.2 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of different types of *M. osloensis* cells pretreated with heat into the shell cavity….77

4.3 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of different types of *M. osloensis* cells pretreated by proteases into the shell cavity……………………………………………………………………………..78

4.4 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection into the shell cavity of different types of *M. osloensis* cells after storage at different temperatures for 2 days with antibiotics ……………………………79

4.5 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection into the shell cavity of different concentrations of purified LPS from 3-day *M. osloensis* cultures………………………………………………………………...80

5.1 Injection, contact and oral toxicity of purified LPS (2 mg/ml) from 3-day *M. osloensis* cultures against *D. reticulatum*……………………………………….101

5.2. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of isolated lipid A moiety (suspended in distilled water or 0.5% (v/v) triethylamine) or polysaccharide moiety (dissolved in distilled water) of *M. osloensis* LPS into the shell cavity………………………………………………………………….102

5.3 Detection of LPS by SDS-PAGE following silver stain………………………..103

5.4 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection into the shell cavity of either galactosamine alone, *M. osloensis* LPS alone, a mixture of galactosamine and *M. osloensis* LPS, or a mixture of galactosamine, *M. osloensis* LPS, and uridine…………………………………………………..104

6.1 Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of 1 to 5-day *M. osloensis* cultures into the shell cavity…………………......……127

6.2 Detection of LPS by SDS-PAGE during culture……………………………………128

6.3 Detection of OMPs by SDS-PAGE during culture………………………………129

6.4 Light micrographs (660 ×) of Gram-stained *M. osloensis* cells during culture...130

6.5 Transmission electron micrographs (46,000 ×) of sections of *M. osloensis* cells during culture…………………………………………………………………...131
6.6 Transmission electron micrographs (210,000 ×) of negatively stained *M. osloensis* pili-like projections from 3-day cultures.................................................................132

7.1 Comparison of sensitivities of two silver staining methods for detecting *M. osloensis* LPS........................................................................................................142

7.2 Comparison of sensitivities of two silver staining methods for detecting *E. coli* J5 or EH100 LPS........................................................................................................143

7.3 Comparison of sensitivities of two silver staining methods for detecting *E. coli* O111:B4 or *S. typhimurium* LPS........................................................................144

7.4 Effect of 20 (A) and 100 (B) min of periodic acid oxidation on sensitivity of the modified silver staining method for detecting *M. osloensis* LPS..............145
CHAPTER 1

INTRODUCTION

Slugs (Mollusca: Gastropoda) are important pests of a wide range of agricultural and horticultural plants in nurseries, home gardens, landscapes, greenhouses, and field crops worldwide (Godan, 1983; South, 1992; Hammond et al., 1999). The gray garden slug \textit{Deroceras reticulatum} (Stylommatophora: Agriolimacidae) is the most common species and often regarded as the most serious slug pest (Wilson et al., 1993; Hammond et al., 1999). Slugs also pose a threat to wildlife and humans because they usually serve as intermediate hosts for many nematode parasites of vertebrates (South, 1992).

Many cultural techniques have been suggested to reduce slug damage, such as increased tillage, varying planting dates, and row cleaners (Hammond and Byers, 2002). However, weather conditions or agronomic factors can preclude the use of cultural measures (Wilson et al., 1993). Chemical control measures (e.g. metaldehyde) may offer acceptable slug control when applied properly. But they are often rendered ineffective under wet and humid conditions that favor slug activity. Furthermore, the molluscicidal
chemicals have unwanted side effects on non-target organisms (South, 1992). Thus,
there is a clear need for more effective and environmentally favorable methods of
controlling slug damage.

Many natural enemies of slugs have been found and studied. They have included
predatory snails (Simmonds and Hughes, 1963; Godan, 1983), Sciomyzidae (Diptera)
(Reidenbach et al., 1989), carabid beetle (Symondson, 1993), slug-parasitic
microsporidian (Jone and Selman, 1985), ciliates (Kozloff, 1956; Brooks, 1968),
trematodes (Foster, 1958; Godan, 1983), cestodes (Abdou, 1958; Valkounova and
Prokopic, 1979), and nematodes (Godan, 1983; Wilson et al., 1993). Phasmarhabditis
hermaphrodita (Rhabditida: Rhabditidae), a bacteria-feeding nematode, was first
described by Schneider (1859) who found the nematodes in decaying terrestrial molluscs.
Maupus (1900) also found the nematodes in the intestine of the slug Arion ater. P. hermaphrodita was originally considered to be non-parasitic to slugs and remain dormant
inside the slug body until the slug died, then grow and multiply on the decaying cadaver
(Maupas, 1900; Mengert, 1953). However, Wilson et al. (1993) indicated that the
nematode actually is an endoparasite of several species of slugs. Up to now, the
nematode has been found to infect and kill a wide variety of pest species of both slug and
snails, as summarized in Table 1.1 (Glen and Wilson, 1997; Speoser et al., 2001; Grewal
et al., 2003). Moreover, the nematode is able to kill slugs living in the soil that are not
susceptible to molluscicidal chemicals applied on the soil surface. Furthermore, it is
effective in wet weather condition and is harmless to other beneficial invertebrates
(Wilson, 1993; Glen and Wilson, 1997) and non-target molluscs (Wilson et al., 2000).
Therefore, P. hermaphrodita has potential for the biological control of mollusk pests.
<table>
<thead>
<tr>
<th>Slugs</th>
<th>Snails</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arion ater</em></td>
<td><em>Cernuella virgata</em></td>
</tr>
<tr>
<td><em>A. distinctus</em></td>
<td><em>Cochlicella acuta</em></td>
</tr>
<tr>
<td><em>A. intermedius</em></td>
<td><em>Helix aspersa</em></td>
</tr>
<tr>
<td><em>A. lusitanicus</em></td>
<td><em>Lymnaea stagnalis</em></td>
</tr>
<tr>
<td><em>A. silvicus</em></td>
<td><em>Monacha cantiana</em></td>
</tr>
<tr>
<td><em>Deroceras caruanae</em></td>
<td></td>
</tr>
<tr>
<td><em>D. laeve</em></td>
<td></td>
</tr>
<tr>
<td><em>D. reticulatum</em></td>
<td></td>
</tr>
<tr>
<td><em>Leidyula floridana</em></td>
<td></td>
</tr>
<tr>
<td><em>Tandonnia budapestensis</em></td>
<td></td>
</tr>
<tr>
<td><em>T. sowerbyi</em></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. Host range of *P. hermaphrodita*. Note that data are from Glen and Wilson (1997); Speoser et al. (2001); Grewal et al. (2003).

As with the entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* (Rhabditida: Heterorhabditidae and Steinernematidae), the parasitic cycle of *P. hermaphrodita* is initiated by the third-stage dauer (enduring or non-aging) juveniles. The dauer juveniles are about 1 mm long with the body diameter of c.a. 40 µm (Hooper et al., 1999). Each of these dauer juveniles is surrounded by a retained second-stage cuticle with closed mouth and anus. Wilson et al. (1993) reported that the dauer juveniles could invade *D. reticulatum* through the dorsal integumental pouch immediately posterior to the mantle and enter the shell cavity via a short canal. Once inside the slug, the dauer juveniles develop into self-fertilizing hermaphrodites, which grow, multiply, and cause
characteristic swelling of the slug mantle, finally resulting in host death within 1-3 weeks (Wilson et al., 1993). When the food source is depleted, the nematodes form a new generation of dauer juveniles that leave the cadaver to search for new hosts.

Unlike *Heterorhabditis* and *Steinernema*, *P. hermaphrodita* has been found to be associated with many different species of bacteria, rather than one particular species (Table 1.2). Wilson et al. (1995b) recovered over 150 bacterial isolates from inside infective juveniles (= dauer juvenile) of *P. hermaphrodita*, from living and dead *D. reticulatum*, and from xenic foam chip cultures of the nematodes. However, nematode yield in *in vitro* cultures and pathogenicity to slugs differ with the different associated bacteria (Wilson et al., 1995b, c). *Moraxella osloensis* was finally selected as the preferred associated bacterium for mass-producing *P. hermaphrodita* in monoxenic culture (Wilson et al., 1995b).

*Moraxella osloensis* is a gram-negative aerobic bacterium related to other members of Moraxellaceae in the gamma subdivision of the purple bacteria. The bacterium is coccal or rod-shaped, but tends to be pleomorphic. *M. osloensis* can grow in mineral media with acetate and ammonium salts, but is sensitive to penicillin. It produces oxidase and catalase, but not indole and pigment. *M. osloensis* is occasionally isolated from the upper respiratory tract, genitourethral specimens, blood, cerebrospinal fluid, and pyogenic manifestations in joints, bursae and other sites from humans (Bovre, 1984). The bacterium is considered as an opportunistic human pathogen, as it has been found to cause human diseases, such as endocarditis (Stryker et al., 1982), osteomyelitis.
(Sugarman and Clarridge, 1982), central venous catheter infection (Buchman et al., 1993), meningitis (Fijen et al., 1994), pneumonia (Vuori-Holopainen et al., 2001), and endophthalmitis (Berrocal et al., 2002).

*P. hermaphrodita* has been mass-produced successfully in monoxenic liquid cultures with *M. osloensis* in fermenters (Wilson et al., 1995b, c). Nematode yield in excess of 100,000 infective juveniles (IJs) per ml has been achieved (Glen and Wilson, 1997). Moreover, the nematode has been field tested in Europe in many crops including winter wheat (Wilson et al., 1994, 1996), lettuce (Wilson et al., 1995a), oilseed rape (Wilson et al., 1995d), strawberry (Glen et al., 1996), and sugar beet (Ester and Geelen, 1998).

<table>
<thead>
<tr>
<th><strong>Family</strong></th>
<th><strong>Species</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonadaceae</td>
<td><em>Aeromonas hydrophila</em></td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas sp.</em></td>
</tr>
<tr>
<td>Bacillaceae</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td><em>Providencia rettgeri</em></td>
</tr>
<tr>
<td></td>
<td><em>Serratia proteamaculans</em></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td><em>Flavobacterium breve</em></td>
</tr>
<tr>
<td></td>
<td><em>F. odoratum</em></td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td><em>Moraxella osloensis</em></td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td><em>Pseudomonas fluorescens</em> (isolate no. 1a)</td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em> (isolate no. 140)</td>
</tr>
<tr>
<td></td>
<td><em>P. fluorescens</em> (isolate no. 141)</td>
</tr>
<tr>
<td></td>
<td><em>P. paucimobilis</em></td>
</tr>
<tr>
<td>Sphingobacteriaceae</td>
<td><em>Sphingobacterium spiritovorum</em></td>
</tr>
</tbody>
</table>

*Table 1.2.* Bacteria associated with *P. hermaphrodita*. Note that data are from Wilson et al. (1995b, c).
1996) where the nematodes gave control equivalent to or superior than the chemical standards. Furthermore, although the nematode takes between 1 and 3 weeks to kill slugs, slug feeding is strongly inhibited within three days of infection thus providing rapid crop protection (Glen et al., 2000; Wilson and Gaugler, 2000).

A commercial product, NemaSlug™, based on the monoxenic culture of *P. hermaphrodita* with *M. osloensis* was launched for sale to UK gardeners in Spring 1994 (Glen et al., 1994). The commercial products are formulated as water-dispersible powders, which are stored under refrigerations. When required, the formulation is mixed with water and applied to soil using conventional spray equipment or a watering can fitted with a hose (Glen and Wilson, 1997). The nematodes are typically applied at a rate of $3 \times 10^9$ IJs per hectare and are usually applied as an overall application to the surface of soil to provide uniform coverage of the entire area. However, Grewal et al. (2001) indicated that application of *P. hermaphrodita* to slug shelters provides more economical control than the overall application to the entire area as slugs possess a well-developed homing behavior with an ability to locate homing sites or shelters from over a meter away and slugs spend significant time in the homing sites during the day as nocturnal foragers. The optimum temperature for the growth of *P. hermaphrodita* is 17°C and it can infect and kill *D. reticulatum* at temperature as low as 5°C (Glen and Wilson, 1997). However, the nematode is sensitive to temperatures above 25°C. Thus, soil temperature at 5 cm depth should be recorded at the time of nematode application, and if possible, daily maximum, minimum and mean soil temperatures should also be recorded. Precautions such as carrying nematodes in a cooler and avoiding direct exposure to sunlight should also be taken when handing and transporting the nematodes to avoid
temperatures above 25°C. *P. hermaphrodita* is also sensitive to desiccation. Therefore, it is better to spray the nematodes onto moist soil and if available, apply up to 5 cm of irrigation afterwards (Wilson and Gaugler, 2000). When the soil surface is dry, efficacy can be enhanced by shallow incorporation of the nematodes into soil (Glen and Wilson, 1997).

However, the virulence mechanism of *P. hermaphrodita* and *M. osloensis* against mollusk hosts is still unknown. Wilson et al. (1995b) reported that a 24 hr culture of *M. osloensis* that was injected into *D. reticulatum* hemocoel was not pathogenic. Pathogenicity of the mass-produced nematodes varies among different batches. Furthermore, the commercial product loses its virulence in storage as its shelf life is only about four weeks under refrigerated conditions. All these factors restrict further development of the product. Therefore, information on the virulence mechanism of the nematode/bacterium complex is necessary to develop a high quality biological control agent that maintains high and stable nematode pathogenicity.

Inspiration about the virulence mechanism of the mollusccidal nematode/bacterium complex to mollusk hosts may be derived from information on the virulence mechanisms of the entomopathogenic nematodes and their associated bacteria toward insect hosts. Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are symbiotically associated with bacteria in the genera *Xenorhabdus* and *Photorhabdus*, respectively, which are harbored in the intestine of the infective juveniles (Bird and Akhurst, 1983; Endo and Nickle, 1991). These non-feeding juveniles invade suitable insect hosts through natural body openings (e.g. mouth, anus or spiracles) (Marcek et al., 1988). Moreover, *Heterorhabditis* spp. are also able to penetrate the
insect’s cuticle from intersegmental areas such as leg and maxilla joints (Bedding and Molyneux, 1982). As the bacteria alone are not able to penetrate the alimentary tract and can not independently enter the host’s hemocoel, the nematodes act as vectors to transport the bacteria into a host in which the latter multiply and kill the host within 24-48 hours (Clarke and Dowds, 1995; Gerritsen et al., 1998; Grewal and Georgis, 1999). Moreover, the bacteria produce antibiotics that inhibit the growth of other microorganisms for preventing the insect cadaver from putrefaction and provide nutrients necessary for nematode survival and reproduction within the dead insect (Akhurst, 1982; Gerritsen et al., 1992). What’s more, the toxicity of the nematode/bacterium complex to insect hosts depends mainly on the symbiotic bacteria. Like many other Gram-negative bacteria, *Xenorhabdus* spp. produce endotoxins. The *X. nematophilus* endotoxins are lipopolysaccharide components of the cell wall that are toxic for the hemocytes of larvae of the wax moth *Galleria mellonella* (Dunphy and Webster, 1988). Exotoxin activity has also been discovered for the bacteria *P. luminescens*, *X. nematophilus* and *X. bovienii* by injecting the bacterial supernatant into insect hosts (Georgis and Kelly, 1997). Bowen and Ensign (1998) found and purified a high-molecular-weight extracellular insecticidal protein complex from *P. luminescens*. The purified toxin complex contains no protease, phospholipase, or hemolytic activity and only a trace of lipase activity, but was found to be active in nanogram concentrations against insects representing four orders of the class *Insecta*. Further, the genes encoding the toxin complex have been cloned. The toxin complex loci *tca*, *tcb*, *tcc*, and *tcd* encode a series of four native complexes. Both the *tca* and *tcb* encode complexes with high oral toxicity to *Manduca sexta* (Bowen et al., 1998). In addition, similar toxin complex gene sequences from *X. nematophilus* have been found
(ffrench-Constant and Bowen, 2000). Therefore, information on this related group of insect-parasitic nematodes and their associated bacteria serves as a solid foundation for exploring the virulence mechanism of the mollusccidal nematode/bacterium complex.

As *D. reticulatum* is a most typical and serious slug pest, the present study was conducted to discern the virulence mechanism of *P. hermaphroditus* and *M. osloensis* to the grey garden slug.

**REFERENCES**


CHAPTER 2

INFECTION BEHAVIOR OF THE RHABDITID NEMATODE

PHASMARHABDITIS HERMAPHRODITA TO THE GRAY GARDEN SLUG

DEROCERAS RETICULATUM

2.1 ABSTRACT

Infection behavior of the rhabditid nematode Phasmarhabditis hermaphrodita to the gray garden slug Deroceras reticulatum was studied. The dauer (enduring or non-aging) juveniles of P. hermaphrodita invade D. reticulatum within 8–16 hr following external exposure, with the posterior mantle region containing the shell cavity serving as the main portal of entry. The dauer juveniles can recover, multiply, and produce new dauer juveniles in the slug and slug feces homogenates, but not in the soil extract. These results demonstrate that P. hermaphrodita is a facultative parasite of the slug and can complete its life cycle under non-parasitic conditions associated with the host. Although the juvenile and adult nematodes can kill the slug if injected into the shell cavity of the host, only the dauer stage can serve as an infective stage in the natural environment.
2.2 INTRODUCTION

Slugs (Mollusca: Gastropoda) are important pests for a wide range of agricultural and horticultural crops throughout the world (Godan, 1983; South, 1992). The gray garden slug *Deroceras reticulatum* is the most common species and often is regarded as the most serious pest (Wilson, et al., 1993). Many parasites of *D. reticulatum* have been found and studied. They have included a microsporidian (Jone and Selman, 1985), ciliates (Kozloff, 1956; Brooks, 1968), trematodes (Foster, 1958; Godan, 1983), cestodes (Abdou, 1958; Valkounova and Prokopic, 1979), and nematodes (Godan, 1983; Wilson et al., 1993). *Phasmarhabditis hermaphrodita*, (Rhabditida: Peloderinae) is a parasite of slugs in the Arionidae, Agriolimacidae, and Milacidae, but is harmless to other beneficial invertebrates, and has been thought to be the most effective biocontrol agent for slugs (Wilson, 1993; Glen & Wilson, 1997).

The parasitic cycle of *P. hermaphrodita* is believed to be initiated by the third-stage dauer (enduring or non-aging) juveniles. Once inside the slug host, the dauer juveniles resume normal growth and develop into self-fertilizing hermaphrodites, finally leading to the death of the slug (Wilson et al., 1993). When the food source is depleted, the nematodes form new dauer juveniles that leave the cadaver in search of new slug hosts. *Phasmarhabditis hermaphrodita* has been found to be associated with several different bacteria rather than one particular species. However, the nematode yield in in vitro cultures and pathogenicity to slugs differs with different species of bacteria (Wilson et al., 1995). Wilson et al. (1995) selected *Moraxella osloensis* (gamma subdivision: Moraxellaceae) as the preferred bacterium for mass-producing *P. hermaphrodita* in monoxenic culture.
However, the infection behavior of *P. hermaphrodita* is still poorly understood. Wilson et al. (1993) suggested that the dauer nematodes could invade *D. reticulatum* through the dorsal integumental pouch, and enter the shell sac via a short canal, then grow and multiply in the shell sac. But they did not report the invading time of the dauer juveniles after external exposure or the body region that is the main portal of entry for the nematodes. Both Maupas (1900) and Mengert (1953) had thought that *P. hermaphrodita* was not a parasite of slugs, and the dauer juveniles remained inside the slug body until the slug died, then reproduced on the dead slug. In contrast, Wilson et al. (1993) reported that the nematode actually was an endoparasite capable of infecting and killing several species of pest slugs. However, they didn’t mention if the nematode is a facultative parasite or not. If so, it is important to check if the dauer stage is the only life stage capable of infecting slugs. Therefore, the present study was designed to answer the following questions: (i) When does *P. hermaphrodita* begin to invade *D. reticulatum* after external exposure? (ii) Which region of the slug body serves as a main entry point for the nematodes? (iii) Can the dauer juvenile recover and multiply in non-parasitic conditions? (iv) Is the dauer stage the only life stage capable of infecting slugs?

### 2.3 MATERIALS AND METHODS

**Source of nematodes and slugs**

Foam or powder formulation of monoxenic culture of *P. hermaphrodita* with its associated bacterium *M. osloensis* was supplied by MicroBio Ltd (Cambridge, U.K.). Nematodes were removed from the formulation by mixing in tap water. Dauer stages were separated from the non-dauer stages by treating the nematode suspension with 5%
of hand soap (AJAX®, Colgate-Palmolive Company, New York, NY) for 5 hr. Nematode dauer stages withstand exposure to detergents, but non-dauer stages do not (Riddle, 1988). The treated suspension was filtered through two layers of tissue paper loaded on an aluminum sieve, which was then placed in a Petri dish containing tap water. The living dauer juveniles migrated through the tissue paper into water and were used in all the following experiments.

All adult *D. reticulatum* were collected from the field and fed on pieces of fresh carrots and cabbage leaves at room temperature for at least 12 days. Only healthy slugs were then used in all the following experiments.

**Time and route of nematode invasion**

When and where the dauer juveniles of *P. hermaphrodita* begin to invade *D. reticulatum* after external exposure was studied. Ten slugs were exposed to 10,000 dauer juveniles on a moist filter paper in a 9-cm-diameter Petri dish. Ten replicate dishes were set up. Ten slugs were placed on a filter paper moistened with tap water in a separate Petri dish as controls. Slugs were fed on pieces of fresh carrots and cabbage leaves at 22°C. At 4, 8, 16, 24, 48 hr after the exposure, one slug was removed from each dish, rinsed in distilled water, killed by brief immersion in boiling water, and dissected to determine the number of nematodes in the following five body sections: head, anterior mantle region, posterior mantle region, posterior to mantle and posterior body region (Figure 2.1). The mantle of the slug was retained prior to dissection. All organs inside the slug body including the alimentary tract were dissected.
Dauer recovery and multiplication in non-parasitic conditions

As *P. hermaphrodita* has been shown to reproduce on a range of bacterial species in the laboratory, our effort was made to determine if the dauer juveniles can recover and multiply in non-parasitic conditions in the soil. Dauer recovery was defined as the conversion of non-feeding third stage juveniles into fourth stages that resume normal growth and development. The dauer juveniles were surface sterilized by immersing in 0.1% Thimerosal for 3 hr. They were then washed 3 times using sterile saline solution (0.85 % NaCl) through a Tyler mesh (45 μm opening). The surface-sterilized dauer juveniles were placed in 42 wells in two 24-well plates with 100 nematodes (in 100 μl sterile saline solution) per well. Each row of six wells was used for one of the following treatments: (i) 900 μl of sterile water was pipetted into each of the six wells used as a
control; (ii) about 10 g soil from a nursery was collected and immersed in 100 ml tap water, stirred, and allowed to settle for 30 min to remove any naturally occurring nematodes or nematode eggs if present in the supernatant, and then 900 µl of soil extract (supernatant) was then added into each of the six wells; (iii) 900 µl of autoclaved soil extract, obtained as above, was pipetted into each of the six wells; (iv) five uninfected *D. reticulatum* were washed with 70% ethanol, ground with 50 ml sterile saline solution using a mortar, and 900 µl of the slug homogenate was then added into each of the six wells; (v) 900 µl of autoclaved slug homogenate, obtained as above, was added into each of the six wells; (vi) about 3 g of slug feces of *D. reticulatum* fed with pieces of fresh carrots and cabbage leaves, were collected and ground with 30 ml of sterile saline solution using a mortar, and 900 µl of the feces homogenate was pipetted into each of the six wells; (vii) 900 µl of the autoclaved slug feces homogenate, obtained as above, was added into each of the six wells. Finally, the two 24-well plates were wrapped with parafilm and incubated at 18°C. At 3, 6, 9, 12, 15 and 18 days after treatments, the numbers of dauer and non-dauer nematodes in each well were counted and the percentage of dauer recovery and total number of the nematodes were calculated.

**Infectivity of different life stages of *P. hermaphrodita* to *D. reticulatum***

As the dauer juveniles recovered and reproduced on slug cadavers and feces, we wanted to find out if non-dauer nematodes also can invade and initiate the parasitic cycle. Therefore, the infectivity of dauer juveniles, juveniles, and adult *P. hermaphrodita*, along with the associated bacterium *M. osloensis* to *D. reticulatum*, was compared using two different methods. Slugs were either exposed to, or injected with, different life stages of
the nematodes. Juvenile and adult nematodes were obtained by exposing ten slugs to 20,000 dauer juveniles on a filter paper in a 9-cm-diameter Petri dish. After 6-7 days of the incubation at 15°C, the slugs were dissected in saline solution (0.85% NaCl) and juvenile and adult nematodes were separated using a microsyringe under a microscope. In the exposure experiment, each slug was exposed to either 50 juveniles, 50 dauer juveniles, or 80 adult nematodes. In the injection experiment, each slug was injected with either 5 juveniles, 5 dauer juveniles, or 8 adult nematodes (in 50 µl saline solution) into the shell cavity using a 1 ml, sterile latex-free syringe. The syringe needle was almost parallel to the slug body and stretched under the slug’s mantle about 2 mm during the injection. Each slug was kept in a separate well on a moist filter paper in a 24-well plate at 15°C for 15 days. Twenty-four slugs filled in a 24-well plate were maintained for each treatment and then divided into four replicates for the calculation of slug mortality, with each row of six slugs in the plate as one replicate. At the same time, slugs injected with the saline solution or exposed to the saline solution served as controls. All slugs were fed on pieces of fresh carrots and cabbage leaves. Dead slugs were dissected to confirm the presence of the nematodes. Total mortality of slugs and percentage of dead slugs infected by the nematodes were recorded.

**Statistical analyses**

All data presented in percentage values were arcsine transformed, and subjected to analysis of variance using a statistical software SYSTAT version 9 (SPSS Inc., 1998). Significant differences among treatments were determined using student’s t-test at \( P = 0.05 \).
2.4 RESULTS

Time and route of nematode invasion

The results of invasion of the dauer juveniles into different regions of the slug are presented in Figure 2.2. Overall, most nematodes were found in the posterior mantle region. No nematodes invaded the slug at 4 hr after the exposure, but at 8-hr exposure, nematodes were found only in the mantle region. Subsequently, more nematodes invaded the mantle region, then began to appear in other body regions by 16 hr. The farther from the posterior mantle, the fewer the nematodes that were found. The nematodes isolated at 48 hr were still in the dauer stage. It seems that the nematodes were associated with the shell sac in the posterior mantle region, but with no particular organs in other sections. The slugs began to die at 3-4 days after the exposure. The dauer nematodes were also found to recover and resume growth at this time. Juvenile nematodes of next generation started to appear 5-6 days following exposure. At 8 days after the exposure, dead slugs were rotten, and hundreds to thousands of juvenile and adult nematodes were in the slug cadaver (not shown). No nematodes were found in the control slugs.

Dauer recovery and multiplication in non-parasitic conditions

The dauer juveniles of *P. hermaphrodita* recovered on autoclaved and non-autoclaved slug homogenate, as well as autoclaved and non-autoclaved slug feces homogenate, but not on the soil extract regardless of whether it was autoclaved or not (Figure 2.3). However, the percentage of dauer recovery on the slug feces homogenate was only about 50%, but was about 80% for the other 3 treatments, 18 days after treatments. The dauer juveniles recovered most quickly in the autoclaved slug feces
homogenate (e.g., recovery was 31.3 ± 1.2 % at 3 days after treatment compared with only 5.8 ± 1.0 %, 3.9 ± 1.9 %, and 1.7 ± 0.8 % for the slug homogenate, autoclaved slug homogenate, and slug feces homogenate, respectively). The percentage of dauer recovery for the autoclaved slug feces homogenate remained the highest during the entire experimental period.

The dauer juveniles also grew and multiplied on the autoclaved or non-autoclaved homogenates of slugs and their feces (Figure 2.4). However, the total numbers of nematodes increased by 12 times in both slug homogenates, but only by about 6 times in the autoclaved slug feces homogenate, and by 1.2 times in the slug feces homogenate, 18 days after treatment. The nematodes stopped multiplying after 9 days in the slug feces homogenate and the autoclaved slug feces homogenate, but continued multiplication for at least 18 days in both slug homogenates.

**Infectivity of different life stages of *P. hermaphrodita* to *D. reticulatum***

There were significant differences (*P* < 0.05) in the infectivity of different *P. hermaphrodita* life stages to *D. reticulatum* after external exposure to the slug host (Figure 2.5). The dauer juveniles caused the highest mortality of slugs, which was about 50%, but the juvenile and adult nematodes did not have significant effect (*P* > 0.05) on the mortality of the slug as compared with the control (saline). Moreover, nematodes were found in the dead slugs that were exposed only to the dauer juveniles. In contrast, all the nematode life stages (juvenile, adult, and dauer nematodes) were pathogenic when
injected directly into shell cavity of the slug (Figure 2.6). Compared to the controls, all life stages resulted in significantly high (> 60%) mortality of slugs ($P < 0.05$). About 95% of the dead slugs were confirmed to have infection of nematodes on dissection.

## 2.5 DISCUSSION

The present results suggest that *P. hermaphrodita* invades *D. reticulatum* within 8-16 hr following external exposure and the posterior mantle region serves as the main entry point. The genital pore of the slug is situated in the right side of the body, just behind the head (Godan, 1983). Therefore, the mouth and genital pore do not appear to be the main portals of entry for the nematodes. These results agree with the observation of Wilson et al. (1993), who suggested that the dauer juveniles invaded the slug host through the dorsal integumental pouch immediately posterior to the mantle, then entered the shell cavity (posterior mantle region) via a short canal. After invasion into the mantle region, the nematodes spread gradually to other body regions, grow, and reproduce by consuming the entire body of the slug. Another parasitic nematode of molluscs, *Cosmocercoides dukae*, has similar infection behavior as *P. hermaphrodita*. Ogren (1959) reported that *C. dukae* was located at the mantle chamber around the vestigial shell in *Deroceras laeve*. And the infective (third-stage) juveniles of *C. dukae* can leave the mantle chamber and infect new hosts, or enter the genital tract of the host and penetrate into the mantle chamber of the developing embryo (Anderson, 1960). Further, Vanderburgh and Anderson (1987) indicated that the infective juveniles entered the genital tract from the mantle chamber by direct penetration of tissues.
Our results also indicate that the dauer juveniles of *P. hermaphrodita* can recover and reproduce on the slug and slug feces homogenates. The nematodes completed their life cycle in these non-parasitic conditions, resulting in the production of next generation of dauer juveniles within 30-40 days (data not shown). *P. hermaphrodita* was originally considered to be non-parasitic to slugs, as the dauer juveniles could enter the slug body and remain dormant until the slug died, then grow and multiply on the decaying cadaver (Maupas ,1900; Mengert, 1953). However, Wilson et al. (1993) indicated that the nematode actually was an endoparasite of several species of slugs. The present results demonstrate that *P. hermaphrodita* is a facultative parasite of *D. reticulatum* as the nematode can complete its life cycle on the living slug host, slug cadaver (regardless of whether it is inside or outside of the cadaver) or slug feces. Stephenson and Knutson (1966) reported that thirty species of nematodes (27 free living) were found to be facultative parasites of *Arion* spp. (Gastropoda: Arionidae). Also, *Tetrahymena rostrata*, a ciliate protozoan, was thought to be a facultative parasite of *D. reticulatum* (Thompson, 1958). Further, the recovery and multiplication of the dauer juveniles in the autoclaved slug homogenate was similar to those in the slug homogenate. This indicates that the bacteria inside the slug may not be critical to the development of the nematodes. However, the nematodes had much lower dauer recovery and multiplication in the slug feces homogenate than in the autoclaved slug feces homogenate, perhaps because of many protozoa present in the slug feces homogenate, which may have competed with the nematodes for food.
The finding that the dauer juveniles of *P. hermaphrodita* can recover and reproduce in slug cadaver or feces may have both negative and positive effects on the use of *P. hermaphrodita* as a biocontrol agent for slugs. For example, the dauer juveniles can resume growth and reproduction in the slug cadaver or feces and thus lose their ability to infect living slugs, resulting in reduced potential for the control of target slugs when used as an inundative biological control agent. However, the recovery and multiplication of the dauer juveniles in the non-parasitic conditions could enable nematodes to persist in the environment in the absence of live hosts. Therefore, *P. hermaphrodita* might be better suited for long-term inoculative slug control than the inundative strategy currently employed.

Only the dauer stage can serve as an infective stage in the natural environment because other life stages did not invade or kill the slug host through external exposure. However, if juvenile and adult nematodes were injected directly into the shell cavity, they were able to cause host death. Therefore, all life stages of *P. hermaphrodita* are pathogenic to *D. reticulatum* once inside the slug. However, compared with non-dauer stages, the dauer juveniles have greater resistance to environmental stresses and thus have a better capacity for surviving, searching and invading the slugs.

### 2.6 ACKNOWLEDGMENTS

We thank Ohio Agricultural Research and Development Center and MicroBio Ltd, Cambridge, U.K. for providing funding for this project and Dr. Ronald Hammond for discussions on slug morphology and biology.
2.7 REFERENCES


Figure 2.2. Time and route of invasion of *P. hermaphrodita* into *D. reticulatum*. Heights of columns and error bars represent mean ± standard error [SE] of 10 replicates.
Figure 2.3. Dauer recovery of *P. hermaphrodita* in non-parasitic conditions. Heights of columns and error bars represent mean ± SE of 6 replicates.
**Figure 2.4.** Multiplication of *P. hermaphrodita* in non-parasitic conditions. Heights of columns and error bars represent mean ± SE of 6 replicates.
Figure 2.5. Infectivity of different life stages of *P. hermaphrodita* to *D. reticualtum* at 15 days after external exposure to the slugs (50 juvenile, 50 dauer, or 80 adult nematodes per slug). Heights of columns and error bars represent mean ± SE of four replicates.
**Figure 2.6.** Infectivity of different life stage of *P. hermaphrodita* to *D. reticulatum* at 15 days after injection into shell cavity (5 juvenile, 5 dauer, or 8 adult nematodes per slug). Heights of columns and error bars represent mean ± SE of four replicates.
CHAPTER 3

PATHOGENICITY OF MORAXELLA OSLOENSIS, A BACTERIUM ASSOCIATED WITH THE NEMATODE PHASMARHABDITIS HERMAPHRODITA, TO THE SLUG DEROCERAS RETICULATUM

3.1 ABSTRACT

Moraxella osloensis, a gram-negative bacterium, is associated with Phasmarhabditis hermaphrodita, a nematode parasite of slugs. This bacteria-feeding nematode has potential for the biological control of slugs, especially the gray garden slug Deroceras reticulatum. Infective juveniles of P. hermaphrodita invade the shell cavity of the slug, develop into self-fertilizing hermaphrodites and produce progeny, resulting in host death. However, the role of the associated bacterium in the pathogenicity of the nematode to the slug is unknown. We discovered that M. osloensis alone is pathogenic to D. reticulatum after injection into the shell cavity or hemocoel of the slug. The bacteria from 60-hr cultures were more pathogenic than the bacteria from 40-hr cultures as indicated by the higher and more rapid mortality of the slugs injected with the former.
Coinjection of penicillin and streptomycin with the 60-hr bacterial culture reduced its pathogenicity to the slug. Further work suggested that reduction and loss of pathogenicity of the aged infective juveniles of *P. hermaphrodita* to *D. reticulatum* results from the loss of *M. osloensis* from the aged nematodes. Also axenic J1/J2 nematodes were non-pathogenic after injection into the shell cavity. Therefore, we conclude that the bacterium is the sole killing agent of *D. reticulatum* in the nematode/bacterium complex, and that *P. hermaphrodita* acts only as a vector to transport the bacterium into the shell cavity of the slug.

### 3.2 INTRODUCTION

*Moraxella osloensis* (gamma subdivision: Moraxellaceae) is a gram-negative aerobic bacterium that is coccal or rod-shaped but which tends to be pleomorphic. The bacterium can grow in mineral medium with acetate and ammonium salts and produces oxidase and catalase but is sensitive to penicillin. *M. osloensis* is occasionally isolated from the upper respiratory tract, genitourethral specimens, blood, cerebrospinal fluid, and pyogenic manifestations in joints, bursae and other sites from human (Bovre, 1984). The bacterium is considered as an opportunistic human pathogen and has been found to cause diseases, such as endocarditis (Stryker et al., 1982), osteomyelitis (Sugarman and Clarridge, 1982), central venous catheter infection (Buchman et al., 1993), meningitis (Fijen et al., 1994), pneumonia (Vuori-Holopainen et al., 2001) and endophthalmitis (Berrocal et al., 2002). *M. osloensis* has also been found to be associated with *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), a lethal nematode parasite of slugs (Wilson et al., 1995b).
This bacteria-feeding nematode has potential for the biological control of mollusk pests, including the gray garden slug *Deroceras reticulatum* (Stylommatophora: Agriolimacidae), which is often regarded as the most serious pest of agricultural and horticultural plants (Godan, 1983; South, 1992; Wilson et al., 1993). As with the entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema*, the parasitic cycle of *P. hermaphrodita* is initiated by the third-stage infective juveniles. The infective juveniles invade *D. reticulatum* through the dorsal integumental pouch immediately posterior to the mantle, and enter the shell cavity via a short canal, and then develop into self-fertilizing hermaphrodites that produce progeny, resulting in host death (Wilson et al., 1993). When the food source is depleted, the nematodes form next generation of infective juveniles, which leave the cadaver to search for new hosts. Unlike *Heterorhabditis* and *Steinernema*, which are associated with one particular bacterium in the genera *Photorhabdus* and *Xenorhabdus*, respectively (Forst and Nealson, 1996), *P. hermaphrodita* has been found to be associated with many different species of bacteria. Wilson et al. (1995a) recovered more than 150 bacterial isolates from inside infective juveniles of *P. hermaphrodita*, from living and dead *D. reticulatum*, and from xenic foam chip cultures of the nematodes, including *M. osloensis*. Nine bacterial isolates were selected and studied, and only two isolates from 24-hr cultures (*Aeromonas hydrophila* and *Pseudomonas fluorescens*, isolate 140) were found to be pathogenic to *D. reticulatum* when injected into the hemocoel of the slug. However, nematodes grown with *M. osloensis* or *P. fluorescens* (isolate 141) were pathogenic, those grown with *Providencia rettgeri* produced inconsistent results, and those grown with *Serratia proteamaculans* and
P. fluorescens (isolate 140) were non-pathogenic to the slug. Finally Wilson et al. (1995a) selected M. osloensis as the preferred associated bacterium to mass-produce P. hermaphrodita in monoxenic culture.

A commercial product, NemaSlug™, based on P. hermaphrodita has been developed in England. However, a high dose of the nematodes (3 × 10⁹ infective juveniles/ha) is required for effective plant protection in the field (Grewal et al., 2001). Pathogenicity of the mass-produced nematodes varies among different batches. Further, aged P. hermaphrodita are less virulent than young ones. All these factors restrict further development of the product. Therefore, information on the virulence mechanism of the nematode/bacterium complex is necessary to develop mass-production systems that maintain high and stable nematode pathogenicity. Availability of a high quality biological control agent is needed to manage slug pests in landscapes, nurseries, and field crops, worldwide.

The present study was conducted to discern the real virulent agent in the nematode/bacterium complex. Wilson et al. (1995a) reported that a 24-hr culture of M. osloensis that is injected into D. reticulatum hemocoel is not pathogenic. As the shell cavity in the posterior mantle region, and not mouth or genital pore, serves as the main portal of entry for P. hermaphrodita (Tan and Grewal, 2001), it is predicted that the nematode carries M. osloensis first into the shell cavity and not the hemocoel. Since important organs, including the kidney, lung and heart, are located in the mantle region, it is possible that M. osloensis alone may kill the slug without entering the hemocoel. Thus,
we hypothesized that *M. osloensis* vectored into the shell cavity could be pathogenic to the slug. We also hypothesized that the pathogenicity of *M. osloensis* may vary with the age and number of the bacteria.

3.3 MATERIALS AND METHODS

**Sources of bacteria, nematodes and slugs**

Pure culture of *M. osloensis* and the foam or powder formulation of monoxenic culture of *P. hermaphrodita* with its associated bacterium *M. osloensis* were supplied by MicroBio Ltd (Cambridge, U.K.). Nematodes were removed from the formulation by mixing them in tap water. Infective stages were separated from the non-infective stages by treating the nematode suspension with a 5% solution of hand soap (AJAX®, Colgate-Palmolive Company, New York, NY) for 5 hr. Nematode infective stages withstand exposure to detergents, but non-infective stages do not (Riddle, 1988). The treated suspension was filtered through two layers of tissue paper loaded on an aluminum sieve. The sieve with the nematodes (on the tissue paper) was then put on a Petri dish containing tap water. The living infective juveniles that migrated through the tissue paper into water were used in all the following experiments.

All adult *D. reticulatum* were collected from the field and fed on pieces of fresh carrots and cabbage leaves at room temperature for at least 12 days. Only healthy adult slugs were then used in the subsequent experiments.
Pathogenicity of *M. osloensis* to *D. reticulatum* after injection into the shell cavity

Two experiments were conducted to determine the pathogenicity of *M. osloensis* to *D. reticulatum*. In the first experiment, the pure culture of *M. osloensis* was inoculated in nutrient agar plates and incubated at 25°C for 40 hr (in log phase). The bacteria were then washed off the plates into a sterile Petri dish using sterile saline solution (0.85% NaCl). The total numbers of bacteria in the suspension were measured with a spectrophotometer with a wavelength at 600 nm and estimated using a standard curve of the bacteria. The bacterial suspensions were then diluted serially into different concentrations, namely, $1.01 \times 10^4$, $1.01 \times 10^5$, $1.01 \times 10^6$, $1.01 \times 10^7$, $1.01 \times 10^8$, $1.01 \times 10^9$, and $1.01 \times 10^{10}$ colony-forming units [CFU]/ml. A 50-µl volume of suspension of each concentration was injected into the shell cavity of *D. reticulatum* as described by Tan and Grewal (2001). Twenty-four slugs were treated with each concentration and were then separated into three Petri dishes (eight slugs per dish) as three replicates for the calculation of slug mortality. Slugs injected with the saline solution served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded every day for 16 days.

Bacteria in stationary phase usually secrete and/or accumulate more toxin(s) than those in log phase (Dewanti and Doyle, 1992; Karunkaran and Devi, 1994; Chaussee et al., 1997). In order to determine whether *M. osloensis* in the stationary phase are more toxic to the slug, we repeated the experiment described above with a 60-hr bacterial culture except that slightly different concentrations of bacteria ($1.23 \times 10^2$, $1.23 \times 10^4$, $1.23 \times 10^6$, $1.23 \times 10^8$, and $1.23 \times 10^{10}$ CFU/ml) were used.
We decided to grow the bacteria on plates rather than in broth culture because (i) it is easier to monitor bacterial contamination on plates than in broth culture, (ii) it is easier to monitor the growth of bacteria on plates than in broth culture as isolated colonies are visible to the naked eye in the third phase of a three-phase streak on a nutrient agar plate for 60-hr bacterial culture, but not for 40-hr bacterial culture, and (iii) culturing the bacteria on plates may eliminate potential adverse effect of broth medium on slug mortality.

Effect of antibiotics on pathogenicity of *M. osloensis* to *D. reticulatum*

Penicillin can interfere with the formation of cell walls of bacteria while streptomycin can block the synthesis of bacterial proteins. The two antibiotics were used to inhibit the growth and metabolism of *M. osloensis*, thus testing their effect on pathogenicity of the bacterium to *D. reticulatum*. Bacterial suspension (1.23 × 10^{10} CFU/ml) from the 60-hr culture was prepared as described above. Fifty microliters of the suspension was injected into the shell cavity of each slug with or without penicillin (500 U/ml) and streptomycin (500 µg/ml) (Sigma Chemical Co., St. Louis, MO). Twenty-four slugs were maintained for each treatment and were then separated into three Petri dishes (eight slugs per dish) as three replicates for the calculation of slug mortality. At the same time, slugs injected with the antibiotics or the saline solution served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded every day for 12 days.
Pathogenicity of *M. osloensis* to *D. reticulatum* after injection into the hemocoel

It is possible that pathogenicity of *M. osloensis* to *D. reticulatum* varies with the age of the bacterial cultures. Therefore, the pathogenicity of cultures of the bacterium of different ages to *D. reticulatum* after injection into the hemocoel was determined. Pure culture of the bacterium was inoculated in nutrient agar plates and incubated at 25°C for 24, 40, and 60 hr, respectively. Bacterial suspensions were prepared, and their concentrations were estimated as described above for the shell cavity injection experiment. The bacteria from the three cultures of different ages were then diluted, and their concentrations were adjusted to $1.0 \times 10^9$ CFU/ml. A 20-µl suspension was injected into the hemocoel from the middle of the hind dorsal portion of each *D. reticulatum* slug. Eighteen slugs were treated with bacteria of each culture age and were then separated into three Petri dishes (six slugs per dish) as three replicates for the calculation of slug mortality. At the same time, slugs injected with 20 µl of sterile saline solution served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded every day for 16 days.

Numbers of viable *M. osloensis* in the fresh and aged infective juveniles of *P. hermaphrodita*

As aged *P. hermaphrodita* are less virulent to slugs, the numbers of viable *M. osloensis* in the infective juveniles from a fresh, a 3-month-old, and an 8-month-old batch were determined. Infective juveniles from the three batches were surface sterilized by immersing in 0.1% Thimerosal for 3 hr. Twenty surface-sterilized infective juveniles were ground using an autoclaved mortar for 30 sec and the nematode homogenate was
plated on a 9-cm-diameter nutrient agar plate. Ten replicates were prepared for each batch. All of the nutrient agar plates were incubated at 25°C for 2 days. Numbers of CFU were then counted.

Pathogenicity of axenic and aged *P. hermaphrodita* to *D. reticulatum*

It was very difficult to culture *P. hermaphrodita* without bacteria (data not shown), however, axenic juvenile nematodes at stage J1 or J2 (J1/J2) were obtained by immersing nematode eggs in 0.1% Thimerosal for 3 hr. They were then transferred, through two changes of the sterile saline solution, to a sterile Petri dish containing sterile saline solution with penicillin (500 U/ml) and streptomycin (500 µg/ml). Five axenic J1/J2 juveniles (n = 3) were ground and the nematode homogenate was then plated on a nutrient agar plate at 25°C for 2 days to verify the axenicity of the nematodes. Infective juveniles from the fresh, 3-month-old, and 8-month-old batches were surface sterilized as described above. Five surface-sterilized infective juveniles from the three batches (only the 8-month-old batch with or without the antibiotics) and 5 axenic J1/J2 juveniles with the antibiotics were injected into shell cavity of the slug. The antibiotics were used to maintain the aseptic environment for the aged or axenic nematodes. Twenty-four slugs were maintained for each treatment and were then separated into three Petri dishes (eight slugs per dish) as three replicates for the calculation of slug mortality. At the same time, slugs injected with the antibiotics or the saline solution served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. Numbers of dead slugs were recorded after 12 days.
Statistical analyses

Data presented in percentage values were arcsine transformed, and subjected to one-way or repeated measures, analysis of variance using the statistical software STATISTICA Kernel Release 5.5 (StatSoft Inc., 2000). Significant differences among treatments were determined using Tukey’s honestly significant difference tests at $P = 0.05$.

3.4 RESULTS

Pathogenicity of *M. osloensis* to *D. reticulatum* after injection into the shell cavity

Pathogenicity of different concentrations of *M. osloensis* from a 40-hr culture to *D. reticulatum* is shown in Figure 3.1. There is a trend that slug mortality increased with the increase of the bacterial concentration over time. Both the highest and second highest concentrations ($5.05 \times 10^8$ and $5.05 \times 10^7$ CFU/slug) of the bacteria had significant effects ($P < 0.05$) on slug mortality at 16 days after treatment compared with the control. Moreover, there is a significant linear relationship ($R^2 = 0.57$) between transformed slug mortality and the log of bacterial concentrations at this time.

The 60-hr bacterial culture was highly pathogenic to the slug after injection into the shell cavity (Figure 3.2). Compared with the control, the highest concentration ($6.15 \times 10^8$ CFU/slug) caused significant slug mortality ($P < 0.05$) as early as 4 day after treatment. The second and third highest concentrations ($6.15 \times 10^6$ and $6.15 \times 10^4$ CFU/slug) also had significant effects on slug mortality at 8 days after treatment. In addition, all of the concentrations of the 60-hr bacterial culture, from 6 CFU per slug to $6.15 \times 10^8$ CFU per slug, resulted in similar and significant slug mortality (around 80%).
at 12 days after treatment. There are significant linear relationships between transformed slug mortality and the log of bacterial concentrations at 4 and 8 days after treatment ($R^2 = 0.54$ and 0.73 for 4 and 8 days, respectively).

**Effect of antibiotics on pathogenicity of *M. osloensis* to *D. reticulatum***

Compared with the two groups of controls, the 60-hr culture of *M. osloensis* with and without the antibiotics, overall, had significant effects ($P < 0.05$) on slug mortality after injection into the shell cavity (Figure 3.3). Both the treatments caused significant slug mortality at 1 day after treatments compared with the results for the controls. After that, coinjection of the antibiotics with the culture reduced pathogenicity of the bacteria to the slug. Only treatment with the bacteria without the antibiotics resulted in slug mortality that differed significantly from that of the controls after 1 day. There were no significant differences ($P > 0.05$) between the results for the two groups of controls (saline and antibiotics treatments).

**Pathogenicity of *M. osloensis* to *D. reticulatum* after injection into the hemocoel**

Since *M. osloensis* from the 60-hr culture were more pathogenic than those from the 40-hr culture, it is possible that the bacteria did not kill the slug just because the 24-hr bacterial culture was non-pathogenic in the study of Wilson et al. (1995a). *D. reticulatum* slug mortality caused by cultures of *M. osloensis* of different ages after injection into the hemocoel is shown in Figure 3.4. Only *M. osloensis* from the 60-hr culture had a significant effect ($P < 0.05$) on slug mortality up to 16 days after treatment compared with the results for the control. In addition, there is a significant linear
relationship ($R^2 = 0.66$) between the total slug mortality after 16 days of treatment and the culture age of *M. osloensis* at this time treatment. The 24-hr bacterial culture had no significant effect ($P > 0.05$) on slug mortality during the entire experimental period.

**Numbers of viable *M. osloensis* in the fresh and aged infective juveniles of *P. hermaphrodita***

The number of viable *M. osloensis* in 20 infective juveniles of *P. hermaphrodita* were $96 \pm 30$ ($\pm$ standard error [SE]) for the fresh batch, $43 \pm 16$ for the 3-month-old batch, and $7 \pm 6$ for the 8-month-old batch. The bacterial numbers between the fresh batch and the 8-month-old batch were significantly different ($P<0.05$). According to the average number of *M. osloensis* per nematode, the infective juveniles were divided into five groups: group 1 (< 1 bacterium/nematode), group 2 (1 to 4 bacteria/nematode), group 3 (5 to 8 bacteria/nematode), group 4 (9 to 12 bacteria/nematode) and group 5 (12 to 15 bacteria/nematode). The percentages of the nematodes from the three batches in the five groups are presented in Figure 3.5. No nematodes from the 8-month-old batch fell in the groups 3 to 5, whereas 10% of the nematodes from the 3-month-old batch and 40% of the nematodes from the fresh batch fell in these groups. In addition, only nematodes from the fresh batch fell in the groups 4-5 (20% and 10% of the nematodes fell in the group 4 and 5, respectively). In contrast, only 20% nematodes from the fresh batch fell in the group 1, whereas 60% of the nematodes from the 3-month-old batch and 90% of the nematodes from the 8-month-old batch belonged to group 1.
Pathogenicity of axenic and aged *P. hermaphrodita* to *D. reticulatum*

Percentage mortality of *D. reticulatum* caused by the axenic and aged *P. hermaphrodita* is shown in Figure 3.6. Only the fresh infective juveniles caused significant slug mortality (*P* < 0.05) compared with the results for the controls. Neither the 8-month-old infective juveniles with or without the antibiotics nor the axenic J1/J2 *P. hermaphrodita* with antibiotics had observable effects on slug mortality.

### 3.5 DISCUSSION

The present results demonstrate that *M. osloensis* alone can kill *D. reticulatum* adults after injection into the shell cavity or hemocoel. Pathogenicity of *M. osloensis*, however, varied with the age of the bacterial cultures: 24 hr cultures were non-pathogenic whereas 40- and 60-hr cultures were pathogenic to the slug. Wilson et al. (1995a) injected only a 24-hr-old *M. osloensis* culture (with unknown concentration) into the hemocoel of *D. reticulatum* and did not observe significant effects on slug mortality after 8 days of treatment. Their results are consistent with what we found when the bacteria of the same culture age were used. However, our results demonstrate that the aged cultures of *M. osloensis* (e.g. 60-hr cultures) are pathogenic to *D. reticulatum* both in the shell cavity and in the hemocoel.

Axenic J1/J2 nematodes were non-pathogenic after injection into the shell cavity and the pathogenicity of the infective juveniles depended on the number of viable bacteria carried by the nematodes. In addition, the 8-month-old batch of the nematodes was almost axenic as only 7 CFU of *M. osloensis* were found in 180 nematodes and these nematodes did not have an observable effect on slug mortality following injection into
the shell cavity. Therefore, we conclude that *P. hermaphrodita* alone is unable to kill the slug host, that the nematode acts only as a vector to transport its associated bacterium, *M. osloensis*, into the shell cavity of *D. reticulatum*. The bacterium appears to be the only killing agent in the nematode/bacterium complex.

Our results strongly suggest that *M. osloensis* may produce a toxin(s) to kill *D. reticulatum*. The 60-hr bacterial cultures were more pathogenic than the 40-hr bacterial cultures, as indicated by the higher and more rapid mortality of slugs injected with the former. It is highly possible that the 60-hr culture, which is in stationary phase, may have produced and/or accumulated more toxin(s) before injection into the shell cavity or hemocoel. Further, reduction in the pathogenicity of the bacteria by the addition of the antibiotics also suggests the involvement of a toxin(s), since the antibiotics may inhibit production of the bacterial toxin(s) by blocking the multiplication and metabolism of *M. osloensis*. Moreover, *M. osloensis* coinjected with the antibiotics caused significant slug mortality, a level of mortality that was the same as that caused by the bacteria injected alone 1 day after treatment, thus suggesting that the toxin(s) may have been produced before the addition of the antibiotics and the antibiotics did not have an effect on the toxicity of the toxin(s). A related bacterium, *Moraxella (Branhamella) catarrhalis*, is regarded as the third most common pathogen of the respiratory tract for humans (Enright and McKenzie, 1997). The liberated endotoxin, histamine and chemotactically active factors are considered as the major pathogenic factors for *M. catarrhalis* (Cullman, 1997). Enright and McKenzie (1997) reported that three serotypes of lipooligosaccharide, fimbriae and a possible capsule might be related to pathogenicity of
the bacterium. Haemagglutinin also might a marker of pathogenicity for *M. catarrhalis* (Fitzgerald et al., 1999). In addition, Hoiczyk et al. (2000) indicated that two adhesins on the outer membrane of *M. catarrhalis* are established pathogenicity factors.

The mutual association between *P. hermaphrodita* and *M. osloensis* seems parallel to the association between the entomopathogenic nematodes and their associated bacteria. The infective juveniles of the entomopathogenic nematodes also vector their symbiotic bacteria *Xenorhabdus* or *Photorhabdus* into insect hosts, in which the latter multiply and kill the hosts within 24 to 48 hours (Grewal and Georgis, 1999). Further, it is the symbiotic bacteria of the entomopathogenic nematodes that produce toxins to kill the insect hosts. Like many other Gram-negative bacteria, *Xenorhabdus* spp. produce endotoxins. The *X. nematophilus* endotoxins are lipopolysaccharide components of the cell wall that are toxic for the hemocytes of *Galleria mellonella* (Dunphy and Webster, 1988). Exotoxin activity has also been demonstrated for *P. luminescens*, *X. nematophilus* and *X. bovienii* by injecting the culture supernatant into insects (Georgis and Kelly, 1997). A high-molecular-weight extracellular insecticidal protein complex in *P. luminescens* has been found and purified. The purified toxin complex contained no protease, phospholipase, or hemolytic activity and only a trace of lipase activity, but was found to be active in nanogram concentrations against insects representing four orders of the class Insecta (Bowen and Ensign, 1998). Further, the genes encoding the toxin complex have been cloned. The toxin complex loci *tca*, *tcb*, *tcc*, and *tcd* encode a series of four native complexes. Both the *tca* and *tcb* encode complexes with high oral toxicity to *Manduca Sexta* (Bowen et al., 1998). Otherwise, similar toxin complex gene sequences from *X. nematophilus* have been found (ffrench-Constant and Bowen, 2000).
It is not fully clear why *M. osloensis* from cultures of different ages had significantly different effects on slug mortality when injected into the shell cavity or hemocoel. As stated above, it is strongly suggested that pathogenicity of *M. osloensis* to the slug is related to the toxicity of the bacterium. As the 24-, 40-, and 60-hr bacterial cultures were in the early log phase, late log phase, and stationary phase, respectively, it is possible that the bacteria from cultures of different ages have different capacities to produce toxin(s) and/or accumulate different amount of toxin(s) before the injection. For example, the 24-, 40-, and 60-hr cultures of *M. osloensis* may have produced and/or accumulated low, medium, and high amount of toxin(s) before injection into the shell cavity or hemocoel, respectively. Furthermore, compared with *M. osloensis* in log phase (e.g. the 24- and 40-hr cultures), the bacterium in stationary stage (e.g. the 60-hr culture) may have specific physiological, biochemical and morphological characteristics enabling better and more rapid slug colonization, evading or overcoming host defense mechanisms.

### 3.6 ACKNOWLEDGMENTS

This work was supported by a Matching Fund Grant from the Ohio Agricultural Research and Development Center and MicroBio Ltd, Cambridge, U.K.

### 3.7 REFERENCES


Figure 3.1. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of different concentrations (CFU/slug) of 40-hr *M. osloensis* culture into the shell cavity. The asterisk indicates results that are significantly different at a *P* of <0.05 from those of the control.
Figure 3.2. Percentage mortality (mean ± SE, n = 3) of D. reticulatum following injection of different concentrations (CFU/slug) of 60-hr M. osloensis culture into the shell cavity. The asterisk indicates results that are significantly different at a $P$ of <0.05 from those of the control.
Figure 3.3. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of *M. osloensis* (60-hr culture) with or without the antibiotics into the shell cavity. Values differ significantly at a $P$ of $< 0.05$ as indicated by different letters.
**Figure 3.4.** Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of cultures of *M. osloensis* of different ages into the hemocoel. Values differ significantly at a $P$ of < 0.05 as indicated by different letters.
Figure 3.5. Percentage of infective juveniles of *P. hermaphrodita* from a fresh, a 3-month-old, and an 8-month-old batch in different groups.
Figure 3.6. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of axenic and aged *P. hermaphrodita* into the shell cavity. IJs, infective juveniles. The asterisk indicates results that are significantly different at a $P$ of $<0.05$ from those of the control.
CHAPTER 4

ENDOTOXIN ACTIVITY OF MORAXELLA OSLOENSIS AGAINST THE GRAY GARDEN SLUG DEROCERAS RETICULATUM

4.1 ABSTRACT

*Moraxella osloensis* is a gram-negative bacterium associated with *Phasmarhabditis hermaphrodita*, a slug-parasitic nematode that has prospects for the biological control of mollusk pests, especially the gray garden slug *D. reticulatum*. This bacteria-feeding nematode acts as a vector to transport *M. osloensis* into the shell cavity of the slug, and the bacterium is the killing agent in the nematode/bacterium complex. We discovered that *M. osloensis* produces an endotoxin(s), which is tolerant to heat and protease treatments and kills the slug after injection into the shell cavity. Washed or broken cells treated with penicillin and streptomycin from 3-day *M. osloensis* cultures were more pathogenic than similar cells from 2-day *M. osloensis* cultures. However, heat and protease treatment, and 2 day of storage at 22°C increased the endotoxin activity of the young broken cells but not the young washed cells treated with the antibiotics. This suggests that there may be a
proteinaceous substance(s) that is structurally associated with the endotoxin(s) and masks its toxicity in the young bacterial cells. Moreover, 2 days of storage of the young washed bacterial cells at 22°C enhanced their endotoxin activity if they were not treated with the antibiotics. Further, purified lipopolysaccharide (LPS) from the 3-day *M. osloensis* cultures was toxic to the slug with an estimated 50% lethal dose of 48 µg per slug, thus demonstrating that the LPS of *M. osloensis* is an endotoxin that is active against *D. reticulatum*. This appears to be the first report of a biological toxin that is active against mollusks.

**4.2 INTRODUCTION**

*Moraxella osloensis* is a gram-negative aerobic bacterium related to other members of Moraxellaceae in the gamma subdivision of the purple bacteria. The bacterium produces oxidase and catalase but not indole and pigment. It is sensitive to penicillin and can grow in mineral media with acetate and ammonium salts (Bovre, 1984). *M. osloensis* is an opportunistic human pathogen as it has been found to cause several human diseases (Stryker et al., 1982; Sugarman and Clarridge, 1982; Buchman et al., 1993; Fijen et al., 1994; Vuori-Holopainen et al., 2001; Berrocal et al., 2002). This bacterium has also been isolated from the xenic foam chip cultures of a lethal nematode parasite of slugs, *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae) (Wilson et al., 1995b).

*Phasmarhabditis hermaphrodita* has potential for the biological control of mollusk pests (Wilson et al., 1993; Grewal et al., 2001), especially the gray garden slug *Deroceras reticulatum* (Stylommatophora: Agriolimacidae) which is the most common
and serious slug pest of agricultural and horticultural plants worldwide (Godan, 1983; South, 1992). *P. hermaphrodita* has been found to be associated with several different species of bacteria. However, nematode yield in in vitro cultures and pathogenicity to slugs differs with different bacterial species (Wilson et al., 1995b). Wilson et al. (1995a) selected *M. osloensis* as the preferred associated bacterium to rear *P. hermaphrodita* in monoxenic culture. A commercial product, NemaSlug™, based on the monoxenic culture of *P. hermaphrodita* with *M. osloensis* has been developed in England.

The parasitic cycle of *P. hermaphrodita* is initiated by the third-stage infective juveniles (IJs). Each of the IJs is enclosed in a retained second-stage cuticle with closed mouth and anus (Wilson et al., 1993). The IJs enter the shell cavity of *D. reticulatum* through the posterior mantle region. Once inside the slug host, the IJs release the associated bacteria (Tan and Grewal, 2001a), resume normal growth and develop into self-fertilizing hermaphrodites, which finally leads to the death of the host (Wilson et al., 1993; Tan and Grewal, 2001a). When the food source is depleted, the nematodes form next generation of IJs that search for new hosts (Wilson et al., 1993). Wilson et al. (1995a) reported that a 24 hr culture of *M. osloensis* that was injected into *D. reticulatum* hemocoel was not pathogenic. However, we (Tan and Grewal, 2001b) discovered that aged cultures of *M. osloensis* were actually pathogenic to *D. reticulatum* both in the shell cavity and in the hemocoel. Moreover, axenic J1 and J2 juveniles of *P. hermaphrodita* were non-pathogenic after injection into the shell cavity, and the pathogenicity of the IJs depended on the number of viable *M. osloensis* carried by the IJs (Tan and Grewal, 2001b). Therefore, it was concluded that *P. hermaphrodita* acts as a vector which transport the associated bacterium *M. osloensis* into the shell cavity of *D. reticulatum* and
that the bacterium is the main killing agent in the nematode/bacterium complex (Tan and Grewal, 2001b). The mutualism between *P. hermaphrodita* and *M. osloensis* seems to be parallel to the association between the entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* and their associated bacteria in the genera *Photorhabdus* and *Xenorhabdus*, respectively (Grewal and Georgis, 1999).

However, the actual mechanism of pathogenicity of *M. osloensis* to *D. reticulatum* is still unknown. We (Tan and Grewal, 2001b) reported that injection of penicillin and streptomycin along with the aged *M. osloensis* cultures reduced the pathogenicity of the bacterium to the slug and suggested that *M. osloensis* may produce a toxin(s) to kill the slug. Information on the pathogenicities of related bacteria in the genus *Moraxella* against their individual hosts inspired us to explore the virulence mechanism of *M. osloensis* against *D. reticulatum*. *Moraxella catarrhalis*, the third most common pathogen of the respiratory tract of humans, is thought to liberate endotoxin for its pathogenicity (Buchman et al., 1993). Moreover, lipopolysaccharide (LPS), outer membrane proteins, pili or fimbriae, and a possible capsule have been considered as virulence factors of *M. catarrhalis* (Verduin et al., 2002). Furthermore, LPS, outer membrane proteins, pili, and the secretion of a hemolysin and/or cytotoxin seem to contribute to virulence of *Moraxella bovis* which is the most common etiological agent isolated in acute and chronic cases of infectious bovine keratoconjunctivitis (Prieto et al., 1999). As endotoxin usually plays a major role in the pathogenesis of gram-negative infections (Enright and McKenzie, 1997), we hypothesized that *M. osloensis* produces an endotoxin(s) that kills the slug. We further hypothesized that the LPS of *M. osloensis* is an endotoxin that is active against the slug.
4.3 MATERIALS AND METHODS

Sources of bacteria and slugs

A pure culture of *M. osloensis* was supplied by MicroBio Ltd (Cambridge, United Kingdom). All *D. reticulatum* adults were collected from the field and fed on pieces of fresh carrots and cabbage leaves at 18°C for at least 12 days. Only healthy adult slugs were then used in the following experiments.

Endotoxin activity of *M. osloensis* from 3-day cultures

A pure culture of *M. osloensis* was inoculated on nutrient agar plates and incubated at 25°C for 3 days until it reached stationary phase. The bacteria were then washed off the plates into a sterile Petri dish by using sterile saline solution (0.85% NaCl). The total numbers of bacteria in the resulting suspension were measured by a spectrophotometer with a wavelength at 600 nm and estimated as $1.58 \times 10^{10}$ colony-forming units [CFU]/ml using a standard curve of the bacteria. Part of the bacterial suspension, which was designated intact cells, was divided into aliquots, placed in 2 ml sterile microcentrifuge tubes, and then centrifuged at 16,000 × g for 5 min using an Eppendorf microcentrifuge (model 5415 C). The obtained supernatant was transferred into a sterile Petri dish and was filtered through a 0.2-µm-pore-size filter. The final filtrate was designated cell-free suspension. The bacterial pellet was then washed with sterile saline solution and was gently pipetted up and down in the tubes to obtain complete suspension following centrifugation at the same conditions. The washing process was repeated three times. The final bacterial pellet was resuspended in the same amount of sterile saline solution as the cell-free suspension and designated washed cells.
A portion of the washed cells was then broken twice using a French pressure cell and press (Thermo Spectronic, Rochester, NY) and the resulting preparation was designated broken cells. Portions (80 µl) of the intact cells, washed cells, with or without penicillin (500 U/ml) and streptomycin (500 µg/ml) (Sigma Chemical Co., St. Louis, MO), broken cells with the antibiotics, and a cell-free suspension were injected into the shell cavities of *D. reticulatum* slugs as described previously (Tan and Grewal, 2001a). The antibiotics were used to inhibit the multiplication and metabolism of the washed cells or of any unbroken cells in the broken cell preparation in order to eliminate the potential production of toxin(s) after injection. Eighteen slugs were used for each treatment and were then separated into three Petri dishes (six slugs per dish) as three replicates for the calculation of slug mortality. Slugs injected with the saline solution or the antibiotics served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded every day for 12 days.

**Effect of heat pretreatment on the endotoxin activities of different types of *M. osloensis* cells**

*M. osloensis* was cultured on nutrient agar plates at 25°C for 2 days (in log phase) and for 3 days (in stationary phase). The bacterial concentrations were estimated and adjusted to $1.0 \times 10^{10}$ CFU/ml. Washed cells or broken cells from the 2 or 3-day cultures were then prepared as described above. Portions of the washed or broken cells were then heated at 60°C or 100°C in a water bath for 15 min. The heated bacterial cells were settled at 22°C for 30 min. An 80-µl suspension of a heated or unheated (at 22°C) preparation of cells with the antibiotics was injected into the shell cavity of each *D.*
The antibiotics were used to inhibit the multiplication and metabolism of the washed cells or of any unbroken cells in the broken cell preparation in order to eliminate the potential production of toxin(s) after injection. The rest of the experimental design was as described above, except that slugs injected only with the antibiotics served as controls.

**Effect of protease pretreatment on the endotoxin activities of different types of *M. osloensis* cells**

Washed or broken cells from 2 or 3-day *M. osloensis* cultures were prepared as described above for the first experiment. Three milliliters of the washed or broken cell preparation was then used to dissolve three trypsin tablets (1 mg of porcine trypsin type II per tablet) containing a small amount of chymotrypsin (Sigma Chemical Co., St. Louis, MO), which yielded a ready-to-use buffered solution (pH 8.0). The obtained mixture was incubated at 25 °C in a water bath for 15 min. An 80-µl portion of the suspension containing the washed or broken cells with the antibiotics was then injected into the shell cavity of each *D. Reticulatum* used. The rest of experimental design was as described above for the first experiment, except that slugs injected with the antibiotics alone or with the proteases plus the antibiotics served as controls.

**Effect of 2 days of storage at different temperatures on the endotoxin activities of different types of *M. osloensis* cells**

Washed or broken cells from 2 or 3-day *M. osloensis* cultures were prepared as described above for the first experiment. The obtained washed or broken cells was
treated with the antibiotics and then divided into 1.5 ml portion, placed into sterile prelabeled micorcentrifuge tubes, and stored for 2 days at 22, 4 or –20°C. The tubes that were stored at 4°C or –20°C were taken out and the contents were allowed to settle at 22°C for 30 min. An 80-µl suspension of a fresh washed or broken cell preparation or a preparation stored at one of the three temperatures for 2 days with the antibiotics was then injected into the shell cavity of each *D. reticulatum* used. The antibiotics were used to inhibit the multiplication and metabolism of the washed cells or of any unbroken cells in the broken cell preparation in order to eliminate the potential production of toxin(s) during the experimental period. The rest of the experimental design was as described above for the second experiment.

**Effect of 2 days of storage at 22°C on the endotoxin activity of washed cells from 2-day *M. osloensis* cultures with or without the antibiotics**

The washed cells from 2-day *M. osloensis* cultures were prepared as described above for the first experiment. Their bacterial concentration was estimated to be 1.32 ×10^{10} CFU/ml before the washing process. The obtained washed cells were divided into two parts, and only one was treated with the antibiotics. Both of the parts were stored at 22°C for 2 days. Eighty microliters of the fresh washed cells or the cells stored at 22°C for 2 days with or without the antibiotics was then injected into the shell cavity of each *D. reticulatum* used. The rest of the experimental design was as described above for the first experiment.
Purification of LPS from 3-day *M. osloensis* cultures

As the endotoxin activity in gram-negative bacteria is generally associated with LPS, we purified the LPS from 3-day *M. osloensis* cultures. Intact cells from the cultures were prepared, centrifuged and washed once as described above for the first experiment. The obtained bacterial pellet was resuspended in distilled water, placed into a sterile beaker, and then lyophilized. LPS was purified from the resulting bacterial powder by classical phenol-water extraction (Westphal and Jann, 1965), with the modification described by Gu et al. (1995). About 0.5 g of the bacterial powder was suspended in 35 ml distilled water at 65-68°C in a water bath. The same amount of 90% phenol, preheated to 65-68°C, was then added with vigorous stirring. The mixture was kept at 65°C for 15 min, and cooled to about 10°C in an ice bath. The resulting emulsion was centrifuged at 3,000 rpm (1470 × g) for 45 min using an IEC clinical centrifuge (model 428). The upper aqueous phase was aspirated off, and the remainder was reextracted with distilled water as described above. Sodium acetate (5 mg/ml) was added to the combined aqueous phase, and LPS was precipitated with 2 volumes of acetone to reduce phospholipid contamination. Pellet was washed twice with 70% ethanol to reduce trace phenol and then suspended in distilled water. RNase and DNase (Sigma Chemical Co., St. Louis, MO) were added (80 µg/ml), and the digestion mixture was incubated at 37°C for 4 hr. Proteinase K (0.5 mg/ml) (Shelton Scientific, Shelton, CO) was then added. The digestion mixture was incubated at 60°C overnight, and then centrifuged twice at 150,000 × g for 3 hr. Gel-like LPS was dissolved in distilled water, lyophilized, and then stored at -20°C. The yield of purified LPS was about 0.7% of dry-weight of the bacteria.
Toxicity of purified LPS from 3-day *M. osloensis* cultures

The purified LPS powder from the 3-day *M. osloensis* cultures was dissolved and diluted with distilled water to produce a series of concentrations of LPS (0.1, 0.4, 0.6, 1.0, 1.6, 2.0 mg/ml). A 50-µl portion of each concentration was injected into the shell cavity of a *D. reticulatum*. Eighteen slugs were treated with each concentration as described above for the first experiment. At the same time, slugs injected with distilled water served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded after 4 days.

Statistical analyses

All data presented in percentage values were arcsine transformed, and subjected to one-way analysis of variance by using the statistical software STATISTICA Kernel Release 5.5 (StatSoft Inc., Tulsa, OK, 2000). Significant differences among treatments were determined using Tukey’s honestly significant difference tests at $P = 0.05$.

4.4 RESULTS

Endotoxin activity of *M. osloensis* from 3-day cultures against *D. reticulatum*

The percentages of mortality of *D. reticulatum* following injection of different components from 3-day *M. osloensis* cultures into the shell cavity are shown in Figure 4.1. Compared with the two groups of controls, all treatments except the cell-free suspension had significant effects ($P < 0.05$) on slug mortality during the entire experimental period. The broken cells with the antibiotics caused the highest level of slug mortality at 1 day after treatment, and the peak mortality was reached on day 4. The
washed cells with the antibiotics had a similar effect on slug mortality, and there was no additional mortality after 4 days. However, the level of slug mortality continued to increase with the intact cells or the washed cells without the antibiotics, reaching 50 and 45%, respectively, by day 12. These results indicate that *M. osloensis* produces an endotoxin(s) that is active against *D. reticulatum*.

**Effect of heat pretreatment on the endotoxin activities of different types of *M. osloensis* cells**

The levels of *D. reticulatum* mortality caused by preheated washed or broken *M. osloensis* cells from the 2 or 3-day cultures are shown in Figure 4.2. Compared with the control (antibiotics treatment), all treatments with the washed or broken cells from 3-day *M. osloensis* cultures caused significant slug mortality (*P* < 0.05). However, the treatments with the washed cells from 2-day *M. osloensis* cultures, regardless of whether the cells were preheated or not, did not cause significant slug mortality (*P* > 0.05) compared with the control. In contrast, the heat pretreatment did have a significant effect on the endotoxin activity of broken cells from the 2-day bacterial cultures. The broken cells from the 2-day cultures did not cause significant slug mortality (*P* > 0.05) when kept at 22°C, but had a significant effect (*P* < 0.05) on slug mortality after they were heated at 100°C for 15 min compared with the results for the control.
Effect of protease pretreatment on the endotoxin activities of different types of *M. osloensis* cells

Protease pretreatment had an effect on the endotoxin activities of different types of *M. osloensis* cells with antibiotics against *D. reticulatum* similar to the effect of the heat treatment described above (Figure 4.3). Compared with the two groups of controls (treatment with antibiotics and treatment with antibiotics plus protease), the washed or broken cells from 3-day *M. osloensis* cultures had significant effects ($P < 0.05$) on slug mortality, but the level of mortality did not increase significantly ($P > 0.05$) after the protease pretreatment. However, the washed cells from 2-day *M. osloensis* cultures did not result in significant slug mortality ($P > 0.05$) during the entire experimental period, regardless of whether the cells were pretreated with the proteases. In contrast, the non- or less-toxic broken cells from the same young cultures, after pretreated with the proteases, had a significant effect ($P < 0.05$) on slug mortality compared with the controls.

Effect of 2 days of storage at different temperatures on the endotoxin activities of different types of *M. osloensis* cells

Similar to the heat and protease treatments described above, the 2 days of storage at different temperatures did not have a significant effect ($P > 0.05$) on the endotoxin activity of the washed or broken cells from 3-day *M. osloensis* cultures against the slugs. However, all treatments with these old bacterial cells caused significantly ($P < 0.05$) higher slug mortality than that of the control (Figure 4.4). Treatment with the washed cells from the 2-day *M. osloensis* cultures, including the fresh washed cells and the cells stored at different temperatures for 2 days, did not have an observable effect on slug
mortality compared with the results for the control. However, the broken bacterial cells from the 2-day cultures stored at 22°C for 2 days resulted in significant slug mortality \((P < 0.05)\) compared with the control.

**Effect of 2 days of storage at 22°C on the endotoxin activity of washed cells from 2 day *M. osloensis* cultures with or without the antibiotics**

If the washed cells from the 2-day cultures were treated with the antibiotics, they had no observable effect on slug mortality after 2 days of storage at 22°C. In contrast, the same washed cells without the antibiotics did not result in significant slug mortality \((P = 0.15)\) when freshly prepared, but caused \(39 \pm 6\%\) (mean ± standard error, \(n = 3\)) slug mortality after storage at 22°C for 2 days, which is significantly different \((P < 0.05)\) from the results obtained for the controls (data not shown).

**Toxicity of purified LPS from three-day *M. osloensis* cultures**

The levels of *D. reticulatum* mortality caused by different concentrations of LPS from 3-day *M. osloensis* cultures are summarized in Fig. 5. There is a significant linear relationship \((R^2 = 0.79)\) between slug mortality and the concentrations of injected LPS. The highest concentration of the LPS (100 µg per slug) caused about 80% slug mortality, whereas less than 6% of the slugs died in the control (distilled water treatment). Furthermore, the 50% lethal dose of the LPS is estimated to be 48 µg per slug by using a log-probit analysis in the statistical software MINITAB Release 13.1 (Minitab Inc., State College, PA, 2000).
4.5 DISCUSSION

The results described here demonstrate that *M. osloensis* produces an endotoxin(s) that kills *D. reticulatum* adults after injection into the shell cavity. The cell-free supernatant of the bacterial suspension was not pathogenic to the slug; thus, no exotoxin activity was detected. However, the washed cells with or without the antibiotics and the broken cells with the antibiotics could kill the slug after injection into the shell cavity, indicating that an endotoxin(s) was produced by the bacterium before injection. Furthermore, the broken cells with the antibiotics resulted in the highest level of slug mortality just 1 day after treatment. It is possible that the intact and washed cells had to be broken by the slug host to liberate the endotoxin(s). In addition, slug mortality caused by the intact cells or the washed cells without the antibiotics increased over time perhaps due to the continued production of the endotoxin(s) resulting from the replication of the bacterial cells inside the slug. We (Tan and Grewal, 2001b) reported previously that 6 and 6.15 × 10^8 CFU of *M. osloensis* per slug resulted in similar significant levels of slug mortality (around 80%) at 12 days after injection into the shell cavity, implying that the live cells of *M. osloensis* may multiply inside the slug after injection.

The present results further demonstrate that the LPS of *M. osloensis* is an endotoxin that is active against *D. reticulatum*. This appears to be the first report on an active biological toxin that is active against mollusks. LPS is an important constituent of the outer membranes of gram-negative bacteria and is resistant to heat and protease treatments. The purified LPS from 3-day *M. osloensis* cultures was toxic to the slug after injection into the shell cavity. Furthermore, dead slugs killed by the LPS exhibited symptoms similar to those exhibited by slugs killed by the intact, washed or broken *M.*
*osloensis* cells. Enright and McKenzie (1997) reported that three serotypes of lipooligosaccharide, fimbriae and a possible capsule might be related to pathogenicity of *M. catarrhalis*. The LPS of *M. catarrhalis* was found to have a effect similar to that of the enterobacterial LPS in mice and in the Limulus amoebocyte lysate assay (Storm et al., 1991). Doyle (1989) reported that formalin-killed isolates of *M. catarrhalis* caused effusions in the middle ear of chinchilla, suggesting the involvement of an endotoxin(s) in the disease process. In addition, the endotoxin produced by *Xenorhabdus nematophilus*, a bacterial symbiont of the entomopathogenic nematode *Steinernema carpocapsae*, is also an LPS that is toxic to the hemocytes of larvae of the wax moth *Galleria mellonella* larvae (Dunphy and Webster, 1988).

Our results suggest that a proteinaceous substance(s) may be structurally associated with the endotoxin(s), which masks its toxicity in the young cells from 2-day *M. osloensis* cultures. Washed or broken cells treated with the antibiotics from the 3-day *M. osloensis* cultures were more pathogenic to the slug than similar cells from the 2-day *M. osloensis* cultures. Furthermore, heat and protease treatment, and 2 days of storage at 22°C increased the endotoxin activity of the young broken cells but not of the endotoxin activity of the young washed cells treated with the antibiotics. As the heat treatments are able to inactivate all proteins in both the young washed cells and the broken cells, the endotoxin activity is unlikely to relate to the biological activity (e.g., enzyme activity) of the proteinaceous substance(s). Therefore, it is possible that the proteinaceous substance(s) structurally associated with the endotoxin(s) may have a physical effect (e.g., steric hindrance) on the endotoxin activity, but is disassociated from the endotoxin(s) by the heat treatment, protease treatment, or storage for 2 days at 22°C in the young broken
cells. However, this did not occur in the young washed cells, perhaps because (i) the endotoxin(s) and the proteinaceous substance(s) were protected from disassociation by other substances around them in the washed cells when treated with heat or storage for 2 days at 22°C or (ii) the proteases could not locate and digest the proteinaceous substance(s), which may not be exposed on the cell surface of *M. osloensis*. In addition, since the LPS is an endotoxin that is active against the slug, the proteinaceous substance(s) may be an outer membrane protein(s) in the cell wall of *M. osloensis*. The enhanced endotoxin activity of the young washed bacterial cells during storage for 2 days at 22°C at the absence of the antibiotics indicates that the young viable bacterial cells are able to increase their endotoxin activity both on plates and in a sterile saline solution. It is possible that the aged bacteria enhance their pathogenicity to the slug by reducing the amount of the proteinaceous substance(s), by decreasing intensity of association between the endotoxin(s) and the proteinaceous substance(s), or by increasing the level of LPS per bacterium. Therefore, the increase of the endotoxin activity of *M. osloensis* cells over time may be related to the structural change of the bacterial cell wall.

### 4.6 ACKNOWLEDGMENTS

This work was supported by a Matching Fund Grant from the Ohio Agricultural Research and Development Center (OARDC) and MicroBio Ltd, Cambridge, U.K. to P. S. Grewal and an OARDC Graduate Research Competitive Grant to L. Tan.
4.7 REFERENCES


Figure 4.1. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of different components of 3-day *M. osloensis* cultures into the shell cavity. Values differed significantly at a *P* of < 0.05 as indicated by different letters.
Figure 4.2. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of different types of *M. osloensis* cells pretreated with heat into the shell cavity. All types of the cells were treated with the antibiotics before injection. Values differed significantly at a *P* of < 0.05 as indicated by different letters. 2-D, 2-day; 3-D, 3-day.
Figure 4.3. Percentage mortality (mean ± SE, \( n = 3 \)) of *D. reticulatum* following injection of different types of *M. osloensis* cells pretreated by proteases into the shell cavity. All types of cells were treated with the antibiotics before the injection. Values differed significantly at a \( P \) of < 0.05 as indicated by different letters. 2-D, 2-day; 3-D, 3-day.
Figure 4.4. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection into the shell cavity of different types of *M. osloensis* cells after storage at different temperatures for 2 days with antibiotics. Values differed significantly at a *P* of < 0.05 as indicated by different letters. 2-D, 2-day; 3-D, 3-day.
Figure 4.5. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection into the shell cavity of different concentrations of purified LPS from 3-day *M. osloensis* cultures. LD$_{50}$, 50% lethal dose.
CHAPTER 5

CHARACTERIZATION OF THE FIRST MOLLUSCIDAL LIPOPOLYSACCHARIDE FROM MORAXELLA OSLOENSIS

5.1 ABSTRACT

*Moraxella osloensis*, a gram-negative bacterium, is found in a mutualistic association with *Phasmarhabditis hermaphrodita*, a slug-parasitic nematode that has potential for the biocontrol of mollusk pests, especially the gray garden slug *Deroceras reticulatum*. This bacteria-feeding nematode vectors *M. osloensis* into the shell cavity of the slug where the bacteria multiply and liberate lipopolysaccharide (LPS) to kill the slug. We discovered that purified *M. osloensis* LPS possessed a lethal injection toxicity but no contact or oral toxicity against *D. reticulatum*. Toxicity of *M. osloensis* LPS resides in the lipid A moiety but not in the polysaccharide moiety. We semiquantitated the LPS as $6 \times 10^7$ endotoxin units per milligram by *Limulus* amebocyte lysate assay. Analysis of the LPS by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver stain revealed that *M. osloensis* LPS is a rough-type LPS with an estimated molecular weight of 5,300. Coinjection of galactosamine with the LPS increased its endotoxin
activity to D. reticulatum by 2-4 fold. The galactosamine-induced sensitization of the slug to M. osloensis LPS was reversed completely by uridine. This appears to be the first report of galactosamine-induced sensitization to LPS in an animal without a liver.

5.2 INTRODUCTION

Moraxella osloensis (gamma subdivision: Moraxellaceae) is a gram-negative aerobic bacterium that can grow in mineral medium with acetate and ammonium salts. The bacterium is usually coccoid or rod in shape, but tends to be pleomorphic. It produces oxidase and catalase and not indole and pigment, but is sensitive to penicillin (Bovre, 1984). The bacterium has been found to cause several human diseases, such as endocarditis (Stryker et al., 1982), osteomyelitis (Sugarman and Clarridge, 1982), central venous catheter infection (Buchman et al., 1993), meningitis (Fijen et al., 1994), pneumonia (Vuori-Holopainen et al., 2001), and endophthalmitis (Berrocal et al., 2002). Thus, it is considered as an opportunistic human pathogen. M. osloensis is also associated with a bacteria-feeding nematode Phasmarhabditis hermaphrodita (Rhabditida: Rhabditidae), which is also a lethal slug-parasitic nematode (Wilson et al., 1995b). This nematode has potential for the biological control of mollusk pests (Wilson et al., 1993; Glen and Wilson, 1997; Grewal et al., 2001), including the gray garden slug Deroceras reticulatum (Stylommatophora: Agriolimacidae), which is the most common and serious slug pest in nurseries, home gardens, landscapes, greenhouses, and field crops worldwide (Godan, 1983; South, 1992; Hammond et al., 1999). P. hermaphrodita has been found to be associated with different bacterial species. However, nematode yield in in vitro cultures and pathogenicity to slugs varies with different species of the associated
bacteria (Wilson et al., 1995a, b). Wilson et al. (1995a) finally selected *M. osloensis* as the preferred associated bacterium to mass-produce *P. hermaphrodita* in monoxenic culture. A commercial product, NemaSlug™, based on the monoxenic culture of *P. hermaphrodita* with *M. osloensis*, was launched in England in 1994 (Glen and Wilson, 1997).

The parasitic cycle of *P. hermaphrodita* is initiated by the third-stage infective juveniles (IJs), which are about 1 mm long with the body diameter of c.a. 40 µm (Hooper et al., 1999). The IJs invade *D. reticulatum* through the dorsal integumental pouch immediately posterior to the mantle, and enter the shell cavity via a short canal in the posterior mantle region (Tan and Grewal, 2001a). Once inside the slug, the IJs develop into self-fertilizing hermaphrodites that grow, multiply, and cause characteristic swelling of the slug mantle, finally resulting in host death within 1-3 weeks. When the food source is depleted, the nematodes form the new generation of IJs that leave the dead slug to search for new hosts (Wilson et al., 1993).

*M. osloensis* was originally considered to be non-pathogenic to *D. reticulatum* because a 24-hr culture of the bacteria was found to be non-lethal after injection into the hemocoel (Wilson et al., 1995a). However, we (Tan and Grewal, 2001b) discovered that aged cultures (e.g., 60-hr cultures) of *M. osloensis* are actually pathogenic to *D. reticulatum* both in the shell cavity and in the hemocoel of the slug. As the nematode alone is unable to kill the slug, we concluded that the nematode only acts as a vector to transport *M. osloensis* into the shell cavity, and the bacterium is the main killing agent in the nematode/bacterium complex (Tan and Grewal, 2001b). We (Tan and Grewal, 2002a) further discovered that *M. osloensis* produces an endotoxin(s), which is tolerant to
heat and protease treatments and kills the slug after injection into the shell cavity. Furthermore, purified lipopolysaccharide (LPS) from 3-day *M. osloensis* cultures is toxic to the slug after injection into the shell cavity, thus demonstrating that *M. osloensis* LPS is an active endotoxin (Tan and Grewal, 2002a). The mutualism between *P. hermaphrodita* and *M. osloensis* is similar to the association between the entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* and their associated bacteria in the genera *Photorhabdus* and *Xenorhabdus*, respectively (Forst and Nealson, 1996), which are the subjects of intensive investigations recently.

*M. osloensis* LPS was found to have a lethal injection toxicity against *D. reticulatum* with an estimated 50% lethal dose of 48 µg per slug (Tan and Grewal, 2002a). However, whether the LPS possesses a lethal contact or oral toxicity needs to be discerned because this is important for direct application of the endotoxin against slugs. Usually, LPS is composed of a lipid A moiety and a polysaccharide moiety, and the toxicity of an LPS resides in the lipid A moiety (Galanos et al., 1977; Dunphy and Webster, 1988). Furthermore, the *Limulus* amebocyte lysate (LAL) assay is a sensitive and powerful test for the detection and semiquantitation of endotoxins (Roth et al., 1989). We (Tan and Grewal, 2002b) reported recently that *M. osloensis* LPS is a rough-type of LPS as only one main band was detected in polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by visualization with silver stain. However, the toxic moiety, LAL activity, and molecular weight of *M. osloensis* LPS are not yet available. Thus, the present study was conducted to further characterize the *M. osloensis* LPS. In addition, Galanos et al. (1979) found that treatment of rabbits, rats, and mice with galactosamine (namely, D-galactosamine-HCl) increased
their sensitivity to the lethal effects of LPS from a bacterium *Salmonella abortus equi* by several thousand folds. The mechanism of enhanced sensitization was thought to be associated with the liver injury caused by galactosamine administration, and this enhanced sensitization to the LPS could be reversed by the administration of uridine, which is known to inhibit the hepatic lesions elicited by galactosamine (Galanos et al., 1979). Since *D. reticulatum* is a lower animal that does not have a liver (Dimitriadis, 2001), we wanted to test whether galactosamine or galactosamine/uridine combination would have similar effect on the susceptibility of *D. reticulatum* to *M. osloensis* LPS as it has on mammals. We hypothesized that galactosamine induces susceptibility of *D. reticulatum* to *M. osloensis* LPS, and this galactosamine-induced sensitization to *M. osloensis* LPS is blocked by administration of uridine.

5.3 MATERIALS AND METHODS

**Sources of bacteria and slugs**

A pure culture of *M. osloensis* was supplied by Becker Underwood Company (formerly MicroBio Ltd), Littlehampton, United Kingdom. All *D. reticulatum* adults were collected from the field and fed on pieces of fresh carrots and cabbage leaves at 18°C for at least 12 days. Only healthy adult slugs were then used in the following experiments.

**Purification and toxicity of *M. osloensis* LPS**

*M. osloensis* LPS was purified from lyophilized bacterial powder from 3-day *M. osloensis* cultures by classical phenol-water extraction (Westphal and Jann, 1965) with
modification as described previously (Gu et al., 1995; Tan and Grewal, 2002b), with a yield of 0.9% of the bacterial dry weight. The purified LPS was then dissolved with distilled water to produce a high concentration of LPS (2 mg/ml). The toxicity of M. osloensis LPS against D. reticulatum was compared using three different methods. The injection toxicity of the LPS was tested by injecting a 50-µl portion of the purified LPS into the shell cavity of each D. reticulatum as described by Tan and Grewal (2001a). Slugs injected with distilled water served as controls for the injection toxicity experiment. The contact toxicity of the LPS was determined by applying 5 µl of the LPS on the hind dorsal portion of the slug. Slugs receiving a 5 µl of distilled water served as controls for the contact toxicity experiment. To test the oral toxicity of M. osloensis LPS, slugs were placed in a Petri dish and fed two pieces of half-dry carrot tubers (2 cm in diameter and 0.5 cm in thickness) that had been treated evenly with the LPS around the top of the carrot tubers (600 µg LPS for six slugs per dish). Slugs fed with the carrot tubers treated with the same amount of distilled water served as controls for the oral toxicity experiment. The average uptake of M. osloensis LPS per slug in the oral toxicity experiment was estimated as 100 µg times the percentage of consumption of the carrot tubers during the experiment. The volume of the tubers was measured by recording the increase of water volume after immersion of the tubers in a 25-ml cylinder containing water before and after the experiment. Slugs were fed pieces of fresh carrots and cabbage leaves in the injection and contact toxicity experiments. There were three replicates each containing six slugs in a Petri dish. All slugs were incubated at 18°C. The numbers of dead slugs were recorded up to 4 days.
Toxic moiety of *M. osloensis* LPS

Lipid A was obtained as a precipitate by hydrolysis of *M. osloensis* LPS (2 mg/ml) with 1% acetic acid at 100°C for 2 hr, followed by centrifugation (16,000 \( \times \) g, 5 min) and successive washing with distilled water and acetone (Masoud et al., 1994). The supernatant from the LPS hydrolysate containing the polysaccharide moiety was centrifuged (150,000 \( \times \) g, 3 hr, 4°C) to remove lipid A and unhydrolysed LPS, and then lyophilized. A 50 \( \mu \)l-portion of the obtained lipid A (resuspended in distilled water or dissolved in 0.5% (v/v) triethylamine (Sigma Chemical Co., St. Louis, MO) to a final volume same as that of the original LPS solution) or the polysaccharide (dissolved in the same amount of distilled water as the original LPS solution) was injected into the shell cavity of each *D. reticulatum*. There were three replicates each containing six slugs in a Petri dish. Slugs injected with distilled water or triethylamine served as controls. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded up to 4 days.

Semiquantitation of *M. osloensis* LPS

An LAL assay was used to detect and semiquantitate the purified *M. osloensis* LPS. The purified LPS was diluted serially with endotoxin-free water (Sigma Chemical Co., St. Louis, MO). Equal volume (0.1 ml) of each LPS concentration and LAL (Sigma Chemical Co., St. Louis, MO) were mixed and incubated at 37°C for 1 hr. The gelation of LAL at the minimal LPS concentration was determined by inverting the mixture. A firm gel was considered a positive reaction (Hochstein et al., 1973). The purified *Escherichia coli* 055:B5 LPS (Sigma Chemical Co., St. Louis, MO) was purchased as a
standard endotoxin to semiquantitate *M. osloensis* LPS. The endotoxin activity in the lipid A or polysaccharide moiety of the LPS was also analyzed by the LAL assay. The sensitivity of the LAL assay is 0.05-0.1 endotoxin units (EU) per ml.

**Molecular weight (Mr) of *M. osloensis* LPS**

LPS preparations were treated for 5 min at 100°C in 0.05 M Tris-HCl buffer (pH 6.8) containing 2% (wt/v) SDS, 10% (wt/v) sucrose and 0.01% bromophenol blue. Twenty microliter of each sample was then loaded on a precast Ready Gel® Tris-HCl polyacrylamide gel (86 × 68 × 1.0 mm) containing 4% and 15% acrylamide in the stacking and separating gels, respectively (Bio-Rad Laboratories, Inc., Hercules, CA). Electrophoresis was performed at 12 mA in the stacking gel and 25 mA in the separating gel until the bromophenol blue had run about 6.7 cm. LPS in the gel was then visualized with the classic silver staining method (Tsai and Frasch, 1982). Commercially available rough-type LPSs from *E. coli* J5 (*Mr* = 4,200) and EH100 (*Mr* = 5,500) (Vaneechoutte et al., 1990) were used as LPS controls and *Mr* markers (Sigma Chemical Co., St. Louis, MO).

**Effect of galactosamine or galactosamine/uridine combination on the susceptibility of *D. reticulatum* to *M. osloensis* LPS**

Sensitivities to LPSs of experimental mammals were enhanced several thousand folds by concurrent injection of the LPSs with galactosamine, and the galactosamine-induced sensitization to LPS was completely reversed by the administration of uridine (Galanos et al., 1979; Silverstein, 1997). Thus, we determined whether administration of
galactosamine or galactosamine/uridine combination (Sigma Chemical Co., St. Louis, MO) have similar effect on the susceptibility of *D. reticulatum* to *M. osloensis* LPS. In preliminary experiments, we determined the lethal and non-lethal concentrations of galactosamine and uridine on slugs. A dose of 0.3 mg galactosamine per slug or 1 mg uridine per slug did not cause any slug mortality, whereas 1 mg galactosamine per slug or 3 mg uridine per slug caused about 30% and 80% mortality, respectively, at 4 day after injection into the shell cavity. The weight of each *D. reticulatum* adult used in these experiments was in the range of 0.6-0.8 g. Furthermore, a mixture of 3 mg uridine and 1 mg galactosamine per slug had no effect on slug mortality, but a mixture of 1 mg uridine and 1 mg galactosamine per slug caused about 30% slug mortality at 4 days after injection. Thus, we used the following treatments: 0.3 mg galactosamine plus 0.01 mg LPS, 0.3 mg galactosamine plus 0.01 mg LPS plus 1 mg uridine, 1 mg galactosamine plus 0.03 mg LPS, or 1 mg galactosamine plus 0.03 mg LPS plus 3 mg uridine per slug. Each mixture (in a 50-µl solution) was injected into the shell cavity of each *D. reticulatum*. Slugs injected with endotoxin-free water (which was used to prepare all chemical solutions used in the experiment), galactosamine alone (0.3 or 1 mg per slug), or LPS alone (0.01 or 0.03 mg per slug), served as controls. There were three replicates each containing six slugs in a Petri dish. All slugs were fed pieces of fresh carrots and cabbage leaves and incubated at 18°C. The numbers of dead slugs were recorded up to 4 days.
Statistical analyses

All data presented in percentage values were arcsine transformed, and subjected to one-way analysis of variance by using the statistical software STATISTICA Kernel Release 5.5 (StatSoft Inc., Tulsa, OK, 2000). Significant differences among treatments were determined using Tukey’s honestly significant difference tests at $P = 0.05$.

5.4 RESULTS

Toxicity of *M. osloensis* LPS

Compared with the results from controls, *M. osloensis* LPS caused significant slug mortality ($P < 0.05$) only when injected into the shell cavity (Figure 5.1). No obvious symptoms were observed for living slugs in the contact or oral toxicity experiment. In the oral toxicity experiment, slugs were found to eat the top half of the carrot tubers (data not shown), thus, the average uptake of the LPS was estimated as 50 µg per slug.

Toxic moiety of *M. osloensis* LPS

Isolated lipid A moiety from the purified *M. osloensis* LPS resulted in slug mortality that differed significantly ($P < 0.05$) from that of the distilled water treatment after injection into the shell cavity, but the polysaccharide moiety did not (Figure 5.2). Unexpectedly, 0.5% (v/v) triethylamine alone was toxic to the slug. However, the lipid A dissolved in the 0.5% triethylamine still caused significantly greater slug mortality ($P < 0.05$) than the triethylamine treatment. In addition, dead slugs killed by the isolated lipid A exhibited symptoms similar to those exhibited by slugs killed by the purified LPS.
Semiquantitation of *M. osloensis* LPS

*M. osloensis* LPS was very active in the LAL assay: 0.1 pg of the LPS (in 0.1 ml of LPS dilution) induced the gelation of LAL, but 0.05 pg LPS did not. Thus, the minimal LPS concentration required for the gelation of LAL was estimated as 1 pg per ml for the *M. osloensis* LPS. Furthermore, 0.06 EU per ml was the minimal concentration for the standard entotoxin (*E. coli* 055:B5 LPS) to cause a positive reaction in the LAL assay. Therefore, *M. osloensis* LPS was semiquantitated to be $6 \times 10^7$ EU/mg. In addition, the isolated *M. osloensis* lipid A moiety also caused a positive reaction in the LAL assay, but the polysaccharide moiety did not.

Molecular weight of *M. osloensis* LPS

Like the two rough-type LPS controls, LPS from *E. coli* J5 or *E. coli* EH100, only one main band was detected in the polysaccharide gel for *M. osloensis* LPS. The Mr of *M. osloensis* LPS was about 5,300 as estimated from the comparison of the electrophoretic mobility of the band representing *M. osloensis* LPS with that of bands representing the two Mr markers (Figure 5.3).

Effect of galactosamine or galactosamine/uridine combination on the susceptibility of *D. reticulatum* to *M. osloensis* LPS

The lethal dose (1 mg/slug) of galactosamine enhanced susceptibility of *D. reticulatum* to *M. osloensis* LPS, and uridine (3 mg/slug) inhibited the sensitization effect of the galactosamine completely (Figure 5.4A). Although 1 mg galactosamine per slug or 0.03 mg LPS per slug caused significant ($P < 0.05$) injection toxicity against the slug
compared with the results for the endotoxin-free water treatment, a mixture of 1 mg galactosamine and 0.03 mg LPS per slug resulted in about 80% slug mortality, which differed significantly \((P < 0.05)\) from that of either galactosamine alone (1 mg/slug) or LPS alone (0.03 mg/slug) treatment. However, a 50 µl portion containing 1 mg galactosamine, 0.03 mg LPS, and 3 mg uridine, when injected into the shell cavity of each *D. reticulatum*, only caused slug mortality similar to that caused by the LPS (0.03 mg/slug) alone treatment.

The sublethal dose (0.03 mg/slug) of galactosamine had an effect on the susceptibility of *D. reticulatum* to *M. osloensis* LPS similar to the effect of the lethal dose (1 mg/slug), and 1 mg uridine per slug blocked the galactosamine-induced sensitization completely (Figure 5.4B). Neither 0.3 mg galactosamine per slug nor 0.01 mg LPS per slug had observable effects on slug mortality after injection into the shell cavity. However, their mixture caused significant \((P < 0.05)\) slug mortality compared with the controls (endotoxin-free water, galactosamine, and LPS treatments). Interestingly, coinjection of 1 mg uridine per slug with the above mixture caused no slug mortality during the entire experiment.

5.5 DISCUSSION

The present results demonstrate that *M. osloensis* LPS possesses a lethal injection toxicity but no contact or oral toxicity against *D. reticulatum* thus implying that it is not feasible to directly apply the endotoxin for slug control. We (Tan and Grewal, 2002a) reported previously that 2 days of storage at 22°C had no detectable effect on the endotoxin activity of broken *M. osloensis* cells from 3-day cultures. Thus, the *M.*
osloensis LPS used here should maintain its toxicity at least for 2 days. *M. osloensis* alone is highly pathogenic to *D. reticulatum* once inside the slug host (Tan and Grewal, 2001b). It is suggested that *P. hermaphrodita* is a necessary natural vector of *M. osloensis* in the application of the nematode/bacterium complex as a biological control agent for slugs.

Our results also demonstrate that the toxicity of *M. osloensis* LPS resides in the lipid A moiety but not in the polysaccharide moiety, which is a common feature for LPSs (Galanos et al., 1977; Dunphy and Webster, 1988). *M. osloensis* LPS was semiquantitated to be $6 \times 10^7$ EU/mg by LAL assay. Furthermore, the LPS from *M. osloensis* is a rough-type LPS with an estimated molecular weight of 5,300. A related bacterium, *Moraxella (Branhamella) catarrhalis*, the third most common pathogen of the respiratory tract of humans, is also thought to liberate LPS during infection (Enright and McKenzie, 1997; Verduin et al., 2002). The LPS of *M. catarrhalis* caused lethal endotoxin activity in D-galactosamine-sensitized C57/BL6 mice, which was comparable to that of enterobacterial LPS (Storm et al., 1991). A 20 pg of purified LPS from *M. catarrhalis* BBH 56 or 80 pg of purified LPS from *M. catarrhalis* CCUG 353 was found to induce the gelation of LAL (Storm et al., 1991). The minimal LPS concentrations required for the gelation of LAL were 10 and 25 pg/ml for purified LPSs from nontypeable *Haemophilus influenzae* strains 2019 and 9274, respectively (Gu et al., 1995). It seems that *M. osloensis* LPS is much more active in the LAL assay than these LPSs described above. LPSs produced by *M. catarrhalis* (Storm et al., 1991), *Moraxella bovis* (Prieto et al., 1999), and *H. influenzae* (Helander et al., 1988; Gu et al., 1995) are also rough-type LPSs as the *M. osloensis* LPS. A rough-type LPS usually contains an
oligosaccharide part linked to lipid A, but lack O-antigenic side chains consisting of repeating oligosaccharide units, which are characteristic of smooth-type LPSs (Galanos et al., 1977; Rietschel et al., 1982). The term lipooligosaccharide (LOS) has also been used for *H. influenzae* LPS (Gilbson et al., 1993). It appears that *M. osloensis* LPS also should be designated as *M. osloensis* LOS.

The results described here demonstrate that galactosamine enhances susceptibility of *D. reticulatum* to *M. osloensis* LPS, and the galactosamine-induced sensitization can be blocked completely by administration of uridine. Galanos et al. (1979) reported that administration of galactosamine in experimental mammals increases their susceptibility to the lethal effects of purified LPS from *S. abortus equi* by several thousand fold, and the sensitization effect is inhibited completely by uridine. They suggested that the mechanisms leading to the sensitization effect are associated with the early metabolic alterations in the liver elicited by the administration of galactosamine (Galanos et al., 1979). Kepper et al (1968) found that intraperitoneal injection of galactosamine in experimental mammals lead to liver injury similar to human viral hepatitis. The galactosamine administration causes a high accumulation of UDP-galactosamine derivatives within 30 min and lead to a depletion of hepatic UTP in the liver, thus inhibiting the biosynthesis of macromolecules (e.g., RNA, proteins and glycogen). Eventually, these alterations result in the damage and death of hepatocytes (Decker and Kepper, 1974). However, the hepatic lesions elicited by galactosamine can be blocked by uridine even at 3h after galactosamine administration (Decker and Kepper, 1972). Stachlewitz et al. (1999) suggested that uridine prevents galactosamine hepatotoxicity not only by rescuing the hepatocyte in the late phases of the injury but also preventing tumor
necrosis factor-alpha release from Kupffer cells, thereby blocking apoptosis that occurs early after galactosamine administration. In addition, many substances have been found to have similar inhibitory or blocking effects on the galactosamine-induced sensitization of experimental mammals to LPS, such as hydrazine sulfate (Silverstein et al., 1991), Boswellic acids (Safayhi et al., 1991), sinomenine (Kondo et al., 1994), matrine (Hu et al., 1996), green tea (He et al., 2001a), coffee (He et al., 2001b), wogonin (Van et al., 2001) etc. It is possible that these substances may also inhibit the galactosamine-induced sensitization of \textit{D. reticulatum} to \textit{M. osloensis} LPS.

It is not fully clear why galactosamine-induced sensitization to LPS also exists in \textit{D. reticulatum} that does not have a liver. Compared with mammals such as rabbit, rat, and mouse, \textit{D. reticulatum} is a lower animal. From an evolutionary perspective, it is reasonable to infer that the mollusk may have some cells or tissues that have very primitive liver-like functions. These cells or tissues may be the potential targets of galactosamine. Compared with the several thousand-fold increase in the sensitization of experimental mammals to LPS by galactosamine, the galactosamine-induced sensitization of \textit{D. reticulatum} to \textit{M. osloensis} LPS is very weak (only 2-4 fold). Since liver is an important and main organ for clearance of endotoxins in mammals (Galanos et al., 1979), it is understandable that liver damage caused by galactosamine dramatically increases the susceptibility of mammals to LPS. Although galactosamine was thought to be a specific hepatotoxic agent whose effect was only confined to the liver (Decker and Keppler, 1974), the involvement of other target organs could not be excluded. As important organs, including the kidney, lung, and heart, are located in the mantle region of \textit{D. reticulatum} (Wilson et al., 1993), it is also possible that galactosamine hurts other
important organ(s) in the slug leading to the induced sensitization of *D. reticulatum* to *M. osloensis* LPS. This appears to be the first report of galactosamine-induced sensitization to LPS in an animal without a liver.

5.6 ACKNOWLEDGMENTS

This work was supported by a Graduate Research Competitive Grant from the Ohio Agricultural Research and Development Center and by a presidential fellowship of the Ohio State University to L. Tan.

5.7 REFERENCES


Figure 5.1. Injection, contact and oral toxicity of purified LPS (2 mg/ml) from 3-day *M. osloensis* cultures against *D. reticulatum*. Heights of columns and error bars represent mean ± SE of three replicates. Values differ significantly at a *P* of < 0.05 as indicated by different letters.
Figure 5.2. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of isolated lipid A moiety (suspended in distilled water or 0.5% (v/v) triethylamine) or polysaccharide moiety (dissolved in distilled water) of *M. osloensis* LPS into the shell cavity. Values differ significantly at a P of < 0.05 as indicated by different letters.
**Figure 5.3.** Detection of LPS by SDS-PAGE following silver stain. Samples of 1 µg were analyzed on SDS-PAGE except as noted. Lane 1 and 2, *M. osloensis* at 1 and 5 µg, respectively; lane 3, *E. coli* EH100; lane 4, *E. coli* J5. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 5.4. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection into the shell cavity of either galactosamine alone, *M. osloensis* LPS alone, a mixture of galactosamine and *M. osloensis* LPS, or a mixture of galactosamine, *M. osloensis* LPS, and uridine. (A) Treatments are: galactosamine (1 mg/slug) alone, *M. osloensis* LPS (0.03 mg/slug) alone, 1 mg galactosamine plus 0.03 mg LPS per slug, and 1 mg galactosamine plus 0.03 mg LPS plus 3 mg uridine per slug; (B) Treatments are: galactosamine (0.3 mg/slug) alone, *M. osloensis* LPS (0.01 mg/slug) alone, 0.3 mg galactosamine plus 0.01 mg LPS per slug, and 0.3 mg galactosamine plus 0.01 mg LPS plus 1 mg uridine per slug. Values differ significantly at a *P* of < 0.05 as indicated by different letters. EFW, endotoxin-free water; G, galactosamine; U, uridine.
CHAPTER 6

TEMPORAL EXPRESSION OF SURFACE EXPOSED ANTIGENS IN
MORAXELLA OSLOENSIS CORRELATES WITH VIRULENCE TO THE SLUG
DEROCERAS RETICULATUM

6.1 ABSTRACT

Moraxella osloensis, a gram-negative bacterium, is a mutualistic symbiont of Phasmarhabditis hermaphrodita, a slug-parasitic nematode that has potential for the biocontrol of mollusk pests, especially the slug Deroceras reticulatum. This bacteria-feeding nematode vectors M. osloensis into the shell cavity of the slug where the bacteria reproduce and liberate lipopolysaccharide (LPS) to kill the slug. We discovered that 1 or 2-day M. osloensis cultures were non or less pathogenic whereas 3 to 5-day M. osloensis cultures were more pathogenic to the slug. The average yield of M. osloensis LPS per bacterium did not differ among the 1 to 5-day cultures. However, M. osloensis cells from the 3-day cultures produced more outer membrane proteins (OMPs) than those from the younger or older cultures. The intensity of M. osloensis aggregation changed with
time of culture, and the bacteria of different ages formed different aggregation patterns. Pili-like projections were rarely present on the surfaces of *M. osloensis* from 1-day cultures, but reached maximal density in 3-day cultures. Isolated pili-like projections were found to adhere to each other to form characteristic patterns. The aggregation of *M. osloensis* cells increased as the density of the projections presented on the bacterial surfaces increased during culture. The temporal expression of the pili-like projections strongly correlates with the temporal pattern of *M. osloensis* virulence to *D. reticulatum*. The changes of *M. osloensis* pathogenicity against *D. reticulatum* during culture strongly correlate with structural changes in the bacterial cell wall.

### 6.2 INTRODUCTION

*Moraxella osloensis* is a gram-negative aerobic bacterium within the family Moraxellaceae in the gamma subdivision of the purple bacteria. The bacterium is coccal or rod shaped, but tends to be pleomorphic. It can grow in mineral medium with acetate and ammonium salts, and can produce oxidase and catalase, but is sensitive to penicillin. *M. osloensis* is occasionally isolated from the upper respiratory tract, genitourethral specimens, blood, cerebrospinal fluid, and pyogenic manifestations in joints, bursae and other sites from human (Bovre, 1984). The bacterium is considered as an opportunistic human pathogen because it has been found to cause several human diseases (Stryker et al., 1982; Sugarman and Clarridge, 1982; Buchman et al., 1993; Fijen et al., 1994; Vuori-Holopainen et al., 2001; Berrocal et al., 2002). *M. osloensis* has also been found to be associated with a bacteria-feeding nematode, *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae), which is also a lethal endoparasite of slugs (Wilson et al.,
The nematode has potential for the biological control of mollusk pests (Wilson et al., 1993; Glen and Wilson, 1997; Grewal et al., 2001), especially the gray garden slug *Deroceras reticulatum* (Stylommatophora: Agriolimacidae) (Godan, 1983; South, 1992; Hammond et al., 1999). Wilson et al. (1995a) recovered more than 150 bacterial isolates associated with *P. hermaphrodita*. However, nematode yield in *in vitro* cultures and pathogenicity to slugs differed with different species of the associated bacteria (Wilson et al., 1995a,b). *M. osloensis* was finally selected as the preferred associated bacterium for mass-producing *P. hermaphrodita* in monoxenic culture (Wilson et al., 1995a). A commercial product, NemaSlug™, based on the monoxenic culture of *P. hermaphrodita* with *M. osloensis* was developed in England in 1994 (Glen and Wilson, 1997).

*M. osloensis* was originally thought to be non-pathogenic to *D. reticulatum* because a 24-h culture of the bacteria was not lethal to the slug after injection into the hemocoel (Wilson et al., 1995a). However, we discovered that the posterior mantle region of *D. reticulatum* containing the shell cavity serves as the main portal of entry for *P. hermaphrodita* (Tan and Grewal, 2001a), and aged cultures of *M. osloensis* are actually highly pathogenic to the slug after injection into the shell cavity or the hemocoel (Tan and Grewal, 2001b). Furthermore, the pathogenicity of *P. hermaphrodita* depended on the number of viable *M. osloensis* carried by the nematodes (Tan and Grewal, 2001b). Therefore, it was concluded that *P. hermaphrodita* vectors *M. osloensis* into the shell cavity of *D. reticulatum*, and the bacterium is the main killing agent in the nematode/bacterium complex (Tan and Grewal, 2001b). We (Tan and Grewal, 2002a) further discovered that *M. osloensis* produces a lethal endotoxin(s) against the slug, which is tolerant to heat and protease treatments. No exotoxins were detected. Purified
lipopolysaccharide (LPS) from 3-day *M. osloensis* cultures was toxic to the slug when injected into the shell cavity with an estimated 50% lethal dose of 48 µg per slug, thus demonstrating that *M. osloensis* LPS is an active endotoxin (Tan and Grewal, 2002a). The purified *M. osloensis* LPS is a rough-type of LPS as only one main band was detected in polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver stain (Tan and Grewal, 2002b). The mutualism between *P. hermaphrodita* and *M. osloensis* is parallel to the association between the entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* and their associated bacteria in the genera *Photorhabdus* and *Xenorhabdus*, respectively (Forst and Nealson, 1996).

We (Tan and Grewal, 2001b) previously reported that intact cells from 60-h *M. osloensis* cultures were more pathogenic to *D. reticulatum* than those from 40-h cultures. Similarly, washed (unbroken) or broken cells treated with penicillin and streptomycin from 3-day *M. osloensis* cultures had higher injection toxicity against the slug than those from 2-day *M. osloensis* cultures (Tan and Grewal, 2002a). Why the aged *M. osloensis* cells are more virulent against *D. reticulatum* is unknown. In a related bacterium, *Moraxella catarrhalis*, which is the third most common pathogen of the respiratory tract of humans (Enright and McKenzie, 1997), surface exposed antigens such as LPS (Enright and McKenzie, 1997; Storm et al., 1991; Verduin et al., 2002), outer membrane proteins (OMPs) (Murphy and Bartos, 1989; Murphy, 1990; Aebi et al., 1998; Lafontaine et al., 2000), and pili (Rikitomi et al., 1997a, b) have been considered as virulence factors. Furthermore, LPS, OMPs, and pili also seem to contribute to virulence of *Moraxella bovis*, which is the most common etiological agent isolated in infectious bovine
keratoconjunctivitis (Lehr et al., 1985; Prieto et al., 1999). However, it is unknown if these bacterial pathogens temporally regulate the expression of the surface exposed antigens. It is possible that OMP(s) and pili-like projections may also act as virulence factors for *M. osloensis* besides the LPS. Therefore, the present study was conducted to discern the possibility of temporal expression of these surface exposed antigens in *M. osloensis* and to determine if the changes in the expression of these antigens will correlate with the changes in the pathogenicity of the bacterium to *D. reticulatum*. Specifically, we investigated daily changes in (i) the pathogenicity of *M. osloensis* to *D. reticulatum*, (ii) average yield of *M. osloensis* LPS per bacterium, (ii) average yield of *M. osloensis* OMPs per bacterium, (iv) *M. osloensis* aggregation behavior using Gram-staining and light microscopy, and (v) the expression of pili-like projections using transmission electron microscopy, for five consecutive days. Our overall hypothesis was that the temporal expression of one or more surface exposed antigens strongly correlates with the temporal pattern of *M. osloensis* virulence towards *D. reticulatum*.

### 6.3 MATERIALS AND METHODS

**Sources of bacteria and slugs**

A pure culture of *M. osloensis* was supplied by Becker Underwood Company (formerly MicroBio Ltd), Littlehampton, U. K. All *D. reticulatum* adults were collected from the field and fed on pieces of fresh carrots and cabbage leaves at 18°C for at least 12 days. Only healthy adult slugs were then used in the following experiments.
Pathogenicity to *D. reticulatum*

Pathogenicity of 1 to 5-day cultures of *M. osloensis* was determined by quantifying slug mortality following the injection of the bacteria into the shell cavity. Pure cultures of *M. osloensis* were inoculated in nutrient agar plates and incubated at 25°C for 1 to 5 days. The bacteria of different ages were then washed off the plates into individual sterile Petri dishes using a sterile saline solution (0.85% NaCl). The total numbers of bacteria in each of five suspensions were measured with a spectrophotometer with a wavelength at 600 nm and estimated using their corresponding standard curve of the bacteria. The bacteria from 1 to 5-day cultures were then diluted, and their concentrations were adjusted to $6.0 \times 10^9$ colony-forming units/ml. An 80-µl portion of each of the suspensions was injected into the shell cavity of *D. reticulatum* as described previously (Tan and Grewal, 2001a). Slugs injected with sterile saline solution served as controls. There were three replicates each containing six slugs in a Petri dish. All slugs were incubated at 18°C. The numbers of dead slugs were recorded up to 4 days.

Average yield of LPS

We tested the possibility that aged *M. osloensis* cells enhance their pathogenicity to the slug by increasing the yield of *M. osloensis* LPS per bacterium during culture. As the LPS profiles of proteinase K-digested whole-cell lysates are similar to those of the homologous purified LPS (Hitchcock and Brown, 1983), the average yield of *M. osloensis* LPS per bacterium was compared among 1 to 5-day cultures using the LPS profiles of proteinase K-digested whole-cell lysates of *M. osloensis*, as described previously (Hitchcock and Brown, 1983) with some modifications. Five bacterial
suspensions from 1 to 5-day *M. osloensis* cultures were prepared, and their concentrations were estimated as described above, respectively. A portion containing $1 \times 10^{10}$ *M. osloensis* cells were then transferred into a 2-ml sterile microcentrifuge tube from each of the five suspensions with different amounts depending on their individual bacterial concentrations, followed by centrifugation at $16,000 \times g$ for 5 min. The obtained bacterial pellet was then resuspended in 1 ml of lysing buffer containing 0.05 M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 4% 2-mercaptoethanol, 10% (wt/vol) sucrose and 0.01% bromophenol blue. The lysate was heated at 100°C for 10 min. Proteinase K (0.5 mg/ml) (Shelton Scientific, Shelton, CO) was then added into each of boiled lysates and incubated at 60°C for 2 h. Twenty microliters of each digested lysates was then loaded on a precast Ready Gel® Tris-HCl polyacrylamide gel ($86 \times 68 \times 1.0$ mm) containing 4% and 15% acrylamide in the stacking and separating gels, respectively (Bio-Rad Laboratories, Inc., Hercules, CA). Electrophoresis was performed at 12 mA in the stacking gel and 25 mA in the separating gel until the bromophenol blue had run about 6.7 cm. LPSs in the gel were then visualized by the classic silver staining method (Tsai and Frasch, 1982). The different amount of purified *M. osloensis* LPSs from 3-day cultures, obtained as described previously (Tan and Grewal, 2002a), were also analyzed on SDS-PAGE as LPS controls and markers.

**Average yield of OMPs**

The average yield of *M. osloensis* OMPs per bacterium was compared among the 1 to 5-day cultures using the OMP profiles in a polyacrylamide gel. *M. osloensis* OMPs were purified as described previously (Murphy and Bartos, 1989) with some
modifications. A bacterial pellet containing $1 \times 10^{10}$ *M. osloensis* cells from 1 to 5-day cultures was obtained as described for the second experiment. The obtained pellet was then suspended in 100 µl of 1 M sodium acetate-0.001M β-mercaptoethanol (pH 4.0). A 900-µl volume of a solution containing 5% Zwittergent Z 3-14 (Sigma Chemical Co., St. Louis, MO) and 0.5 M CaCl$_2$ was added. The mixture was then allowed to settle at room temperature for 1 h. Nucleic acids were precipitated by the addition of 250 µl of cold ethanol and subsequent centrifugation at 17,000 $\times$ g for 10 min at 4°C. The supernatant was added with 3.75 ml of cold ethanol, followed by centrifugation at 17,000 $\times$ g for 20 min at 4°C. The obtained pellet was allowed to air dry and then suspended in 300 µl of buffer Z containing 0.05% Zwittergent Z 3-14, 0.05 M Tris, and 0.01 M EDTA (pH 8.0). The mixture was then settled at room temperature for 1 h. The OMPs were present in the buffer Z soluble fraction after centrifugation of the mixture at 12,000 $\times$ g for 10 min at 4°C. The obtained OMP preparations were treated for 5 min at 100°C in 0.1 M Tris-HCl buffer (pH 6.8) containing 2% (wt/v) SDS, 10% (wt/v) glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue. Twenty microliter of each OMP preparation and 5 µl of protein molecular marker SigmaMarker™ (Sigma Chemical Co., St. Louis, MO) were analyzed on SDS-PAGE as described for the second experiment. Proteins in the gel were then stained by gently shaking the gel in a solution (Sigma Chemical Co., St. Louis, MO) containing 0.25% (w/v) Brilliant blue R, 40% (v/v) methanol and 7% (v/v) acetic acid for 6 h, followed by successive destaining with 7% (v/v) acetic acid.
**Aggregation behavior**

Light micrographs of Gram-stained *M. osloensis* Cells from 0 to 5-day cultures were compared to check whether *M. osloensis* cells tended to aggregate and whether the intensity and/or pattern of the bacterial aggregation changed with time of culture. Pure cultures of *M. osloensis* were inoculated and incubated for 1 to 5 days as described for the first experiment. One isolated colony from each of the 0 to 5-day cultures was picked up and applied to a small drop of water on a clear glass slide to yield a thin, uniform smear. The smear was allowed to air dry and was then heat-fixed by passing the slide through a low flame 2 to 3 times. The fixed smear was Gram-stained as described previously (Jones, 1991). The stained smear was examined under a light microscope with a total magnification of 1,000 times.

**Viewing of pili-like projections on the cellular surface**

*M. osloensis* cells from 1 to 5-day cultures were processed for transmission electron microscopy to determine whether *M. osloensis* possessed pili-like projections and whether the density of the projections changed with time of culture. *M. osloensis* cells from 1 to 5-day cultures were fixed in 3% glutaraldehyde and 1% paraformaldehyde in 0.1M potassium phosphate buffer (pH 7.4) 16 h at 4°C. Fixed cells were spun and embedded in 0.8% agarose blocks (Agarose 1, Amresco). Samples were then washed three times with 0.1M potassium phosphate buffer (pH 7.4), postfixed in 2% osmium tetroxide in 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at room temperature, washed three time with distilled water, and dehydrated using a graded ethanol and acetone series. Samples were embedded in Spurr's resin following manufacturer
instructions (Spurr, EMS). Ultrathin sections were counterstained with 3% uranyl acetate in 50% ethanol for 35 min and 2% aqueous bismuth sulfate stain for 15 min, and viewed on a Hitachi H-7500 transmission electron microscope. Ten bacterial sections from each culture were randomly selected. The numbers of pili-like projections for each section were then counted.

**Isolation of pili-like projections from 3-Day *M. osloensis* cultures**

As aged *M. osloensis* cells were found to possess pili-like projections, the projections were isolated from 3-day *M. osloensis* cells by a method (Gerlach and Clegg, 1988) that was previously designed for the isolation of type III pili (whose length was similar to those of the pili-like projections) with some modifications. The *M. osloensis* suspension from 3-day cultures was prepared as described for the first experiment. The suspension was divided into 50 aliquots, placed in 2-ml sterile microcentrifuge tubes, and then centrifuged at 20,800 × g for 10 min to pellet the bacterial cells. The obtained pellet in each tube was suspended in 0.2 ml of sterile saline solution (0.85% NaCl), and then homogenized using a Dremel® cordless rotary tool (model 770) (Dremel, Racine, WI) at 15,000 rpm for 1 min, followed by centrifugation at 20,800 × g for 10 min. The obtained supernatants were collected in a sterile beak. Solid ammonium sulfate (10%, w/v) was added into the supernatant with stirring, and then allowed to settle at room temperature for 30 min, followed by centrifugation at 20,800 × g for 30 min. The new supernatants were pooled and added with an additional 30% (w/v) of solid ammonium sulfate with stirring. Precipitation was allowed to proceed at 4°C for 2 h. Gel-like pellet was then obtained by centrifugation at 104,000 × g for 1 h, and resuspended in distilled water.
Morphology of the isolated pili-like projections

The gel-like pellets containing the isolated pili-like projections were observed with the transmission electron microscope following negative staining. The projection preparations were placed onto a 300 mesh Formvar and carbon-coated copper grids and stained using 1% (w/v) phosphotungstic acid (pH 7.4) for 5 min. The grids were then examined with a Hitachi H-7500 transmission electron microscope. In addition, pellets obtained by the first solid ammonium sulfate (10%, w/v) treatment also were examined to check the potential presence of the pili-like projections as described above.

Statistical analyses

Data presented in percentage values were arcsine transformed, and subjected to one-way analysis of variance by using the statistical software STATISTICA Kernel Release 5.5 (StatSoft Inc., Tulsa, OK, 2000). Significant differences among treatments were determined using Tukey’s honestly significant difference tests at \( P = 0.05 \).

6.4 RESULTS

Pathogenicity to D. reticulatum

Compared with the results from the controls (saline treatment), 1- or 2-day *M. osloensis* cultures did not cause significant \( (P > 0.05) \) slug mortality after injection into the shell cavity. However, 3 to 5-day *M. osloensis* cultures resulted in slug mortality that differed significantly \( (P < 0.05) \) from that of the controls (Figure 6.1). In addition, there
was a significant linear relationship ($R^2 = 0.57$) between the slug mortality and the culture age of *M. osloensis* for the first three days of cultures. It seems that the 3-day *M. osloensis* culture is most pathogenic against *D. reticulatum*.

**Average yield of LPS**

The average yield of *M. osloensis* LPS per bacterium did not differ among the 1 to 5-day cultures (Figure 6.2). Compared with the density of bands representing the LPS controls (the purified LPS from 3-day cultures in lane 6 and 7), the LPS amount in each of the 20 µl of loaded samples was estimated to 1 µg. Thus, the average yield of LPS in the 1 to 5-day cultures was estimated to $5 \times 10^{-15}$ g per bacterium. The molecular weight of the purified *M. osloensis* LPS had been estimated as 5,300 (Tan, L. and Grewal, P. S. (2002) *J. Biol. Chem.* in review). Based on the Avogadro’s number ($6.022 \times 10^{23}$), the number of *M. osloensis* LPS molecules was further estimated to $5.68 \times 10^5$ LPS molecules per bacterium.

**Average yield of OMPs**

*M. osloensis* cells from 3-day cultures had the highest average protein yield per bacterium for all OMPs detected on the gel, however, the average yield of *M. osloensis* OMPs per bacterium did not differ among the other four cultures (Figure 6.3). Totally 15-20 OMPs were detected on the gel with two of these proteins predominating. The molecular weights of the OMPs ranged from 10 to 200 Kda and the two main OMPs were ca. 36 and 45 KDa, respectively.
**Aggregation behavior**

*M. osloensis* cells from 0 to 5-day cultures tended to aggregate, and the intensity of the bacterial aggregation changed with time of culture. *M. osloensis* cells of different culture ages also formed different aggregation patterns (Figure 6.4). *M. osloensis* cells from inoculums (0-day cultures) or 5-day cultures formed an archipelago-like pattern (Figure 6.4A and F). It appears that newborn *M. osloensis* cells in the 1-day cultures separate themselves from the others (Figure 6.4B). Interestingly, the bacteria from the 2-day cultures tended to adhere to each other and formed a loose net-like pattern (Figure 6.4C). Furthermore, *M. osloensis* cells from 3-day cultures formed a tight net-like pattern (Figure 6.4D). The bacteria began to separate gradually after 3 days (Figure 6.4E and F).

**Viewing of pili-like projections on the cellular surface**

The numbers of pili-like projections per section were 0.7 ± 0.3 (mean ± standard error [SE], *n* = 10) for the 1-day cultures, 7.0 ± 1.4 for the 2-day culture, 63.2 ± 1.9 for the 3-day culture, 25.9 ± 5.2 for the 4-day culture, and 18.5 ± 4.7 for the 5-day culture. Pili-like projections were rarely present on the surfaces of *M. osloensis* cells from 1-day cultures (Figure 6.5A). However, they began to appear on the surfaces of the bacteria in 2-day cultures (Figure 6.5B), and reached maximal density in the 3-day cultures (Figure 6.5C). Some *M. osloensis* cells from 4 or 5-day cultures retained the pili-like projections on their surfaces but with a lower density (Figure 6.5D and E), and a few bacteria in the 4 or 5-day cultures were found to completely lose the projections (data not shown). In addition, the pili-like projections, which measured 7-10 nm in diameter and up to ca. 100 nm in length, were peritrichously distributed and needle shaped.
Morphology of the isolated pili-like projections

The isolated pili-like projections from 3-day *M. osloensis* cultures had similar diameter and length to those attached to the bacterial surfaces (Figure 6.6), but were arc-like in sharp (Figure 6.6A). In addition, the isolated projections were found to adhere to each other to form characteristic patterns. For example, two projections adhered together to form a “C”-like structure or a circle (Figure 6.6B), and three projections formed a big circle (Figure 6.6C). In addition, the pili-like projections were not found in the pellets obtained by the first solid ammonium sulfate (10%, w/v) treatment.

6.5 DISCUSSION

The results described here demonstrate that pathogenicity of *M. osloensis* cultures against *D. reticulatum* changes with time of culture: 1 or 2-day *M. osloensis* cultures were non or less pathogenic to the slug whereas 3 to 5-day *M. osloensis* cultures were highly pathogenic after injection into the shell cavity. The results were similar when bacteria of different ages were injected into the slug hemocoel (Tan and Grewal, 2001b). Our results indicate that the average yield of *M. osloensis* LPS per bacterium did not change during culture, and thus have no relationship with observed differences in the pathogenicity of *M. osloensis* cultures to the slug. We (Tan and Grewal, 2002a) previously suggested that a proteinaceous substance(s) may be structurally associated with the *M. osloensis* LPS and mask its endotoxin activity in 2-day *M. osloensis* cultures. Fiske et al (2001) reported that ubiquitous surface protein (Usp)A2, an OMP of *M. catarrhalis*, was very tightly associated with *M. catarrhalis* LPS so that they could not be dissociated with Triton X-100. Since surface proteins related to UspAs (including
UspA1 and UspA2) are found to be widespread among proteobacteria including human pathogenic and environmental species (Hoiczyk et al., 2000), it is possible that an OMP(s) of *M. osloensis* related to UspA2 is structurally associated with *M. osloensis* LPS and masks its endotoxin activity in the young cultures. The present results show that *M. osloensis* cells from 3-day cultures produced more OMPs than those from the younger or older *M. osloensis* cultures. Thus, it seems that the aged bacteria enhance their pathogenicity to the slug not by reducing the production of the OMP(s), either. However, it does not rule out the possibility that the aged bacteria enhance their pathogenicity to the slug by decreasing the intensity of association between the LPS and the OMP(s).

The present results also demonstrate that aged *M. osloensis* presents pili-like projections on its cellular surface. The aggregation of *M. osloensis* cells increased as the density of the projections presented on the bacterial surfaces increased during culture thus suggesting that the projections are responsible for the intercellular adherence. Furthermore, The temporal expression of the pili-like projections strongly correlates with the temporal pattern of *M. osloensis* virulence to *D. reticulatum*: the non or less pathogenic 1 or 2-day *M. osloensis* cultures had no or few pili-like projections whereas the 3-day culture, which seemed to be most pathogenic to the slug, possessed the highest density of the projections, moreover, the 4 or 5-day cultures retained pathogenic to the slug perhaps because that 40-60% of *M. osloensis* cells in the cultures still presented relatively high density (ca. 35 projections per section) of the pili-like projection. The surface projections of bacterial pathogens are usually used for attachment to host cells (Beachey, 1981), it is suggested that the pili-like projections enable better or more rapid slug colonization thus playing an important role in the pathogenicity of *M. osloensis*
against *D. reticulatum*. Rikitomi et al. (1997a) reported that the adherence of *M. catarrhalis* to nasopharyngeal epithelial cells was reduced by denaturing *M. catarrhalis* pili or by using anti-pili antibodies. Furthermore, piliated *M. catarrhalis* was found to bind more efficiently to lower bronchial epithelial cells than nonpiliated bacteria (Rikitomi et al., 1997b). In addition, piliated *M. bovis* was pathogenic after inoculation into the eyes of calves, but nonpiliated variants of the same strain were not (Pedersen et al., 1972).

The overall morphology of the pili-like projections on *M. osloensis* surfaces is similar to that of pili observed on other pathogenic bacteria. As the isolated projections were similar in size to those presented on the bacterial surfaces after separation from *M. osloensis* by the method, which was previously designed for isolation of type III pili of *Klebsiella pneumoniae* (Gerlach and Clegg, 1988), the projections are unlikely to be non-pilus-associated adhesins, which are monomeric or oligomeric proteins anchored to the outer membrane (Hultgren et al., 1993; Hoiczyk et al., 2000). The pili-like projections are peritrichous and needle-like appendages with 7-10 nm in diameter and up to 100 nm in length. These projections are considerably thinner than flagella that have a diameter of about 20 nm (Silverman and Simon, 1977), but are much thicker than curli which are wiry fibers with ca. 2 nm in diameter (Olsen et al., 1989). The diameter of the *M. osloensis* projections is thicker than that of type III pili (Gerlach and Clegg, 1988), but is comparable to that of other types of bacterial pili reported (Orndorff and Falkow, 1984; Klemm, 1985; Ruehl et al., 1988). Furthermore, the projection’s length is similar to that of type III pili (Gerlach and Clegg, 1988), but the projection is much shorter than other types of bacterial pili reported. Type I pili of *Escherichia coli* and other Gram-negative
enteric bacteria were found to be about 2 µm long (Brinton, 1965). Sajjan et al. (1995) reported cable-like type II pili of Burkholderia (Pseudomonas) cepacis, which were 2 to 4 µm in length. In addition, type IV pili of M. bovis were measured up to 3 µm (Ruehl et al., 1988). Interestingly, other related bacteria in the genus Moraxella, including M. bovis (Ruehl et al., 1988), M. catarrhalis (Marrs and Weir, 1990), and M. nonliquefaciens (Froholm and Sletten, 1977) have been found to possess type IV pili which are long filaments extending from the poles of the bacteria. Furthermore, hybridization test with a M. bovis Q pilin (type IV) gene showed that DNA sequences similar to the Q pilin gene are present in M. osloensis (Marrs and Weir, 1990). However, projections similar to type IV pili were not found for M. osloensis in the present study.

Our results also suggest that M. osloensis regulates the expression of the pili-like projections programmatically during culture. The regulation seems to be related with the growth phases of the bacterium. For example, the 1-, 2- and 3-day bacterial cultures were in the early log phase, late log phase, and stationary phase, respectively, whereas the 4- and 5-day cultures might be close to or have been in the death phase. It is possible that M. osloensis focuses on multiplication without producing the projections when nutrients are plentiful (e.g., in the early log phase), but begins to arm itself with the projections when nutrients are nearing depletion (e.g., in the late log phase), eventually resulting in the highest density of the projections when nutrients are completely depleted (e.g., in the stationary phase). In addition, the projections on the dead bacterial surfaces might be destroyed or digested by substances around them (e.g. exoproteinases).

In brief, surface exposed antigens, including LPS, OMP(s), and pili-like projections may be virulence factors for M. osloensis as in other pathogenic bacteria. The
Aged bacteria appear to enhance their pathogenicity to *D. reticulatum* during culture neither by increasing the production of the LPS nor by reducing the production of the OMP(s), but most probably by developing and increasing the density of the pili-like projections on their surfaces and by decreasing the intensity of association between the LPS and the OMP(s). Since the LPS, OMP(s), and pili-like projections all are located in the bacterial cell wall, the changes of the pathogenicity of *M. osloensis* against *D. reticulatum* during culture strongly correlate with structural changes in the bacterial cell wall.

### 6.6 ACKNOWLEDGMENTS

This work was supported by a Graduate Research Competitive Grant from the Ohio Agricultural Research and Development Center and by an Ohio State University presidential fellowship to L. Tan. We thank Ms. Karli Fitzelle and Mr. Dave Fulton for providing help on the preparation of samples for transmission electron microscope.

### 6.7 REFERENCES


Figure 6.1. Percentage mortality (mean ± SE, n = 3) of *D. reticulatum* following injection of 1 to 5-day *M. osloensis* cultures into the shell cavity. Values differ significantly at a *P* of < 0.05 as indicated by different letters.
Figure 6.2. Detection of LPS by SDS-PAGE during culture. Proteinase K-digested lysates of $1 \times 10^{10}$ *M. osloensis* cells were analyzed on SDS-PAGE except as noted. Lane 1 to 5: 1 to 5-day cultures, respectively; Lane 6 and 7: purified *M. osloensis* LPS from 3-day cultures at 1 and 2 µg, respectively. Note that the faint bands on the upper part of the gel might represent undigested proteins in the digested mixtures. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 6.3. Detection of OMPs by SDS-PAGE during culture. Purified OMPs from $1 \times 10^{10}$ *M. osloensis* cells were analyzed on SDS-PAGE except as noted. Lane 1: protein molecular weight markers (Note that the unit of molecular weight is KDa); Lane 2 to 6: 1 to 5-day cultures, respectively. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 6.4. Light micrographs (660 ×) of Gram-stained *M. osloensis* cells during culture. A to F represent *M. osloensis* cells from 0 to 5-day cultures, respectively. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 6.5. Transmission electron micrographs (46,000 ×) of sections of *M. osloensis* cells during culture. A to E represent an *M. osloensis* cell from 1 to 5-day cultures, respectively. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 6.6. Transmission electron micrographs (210,000 ×) of negatively stained *M. osloensis* pili-like projections from 3-day cultures. (A) single arc-like projection; (B) two projections forming a “C”-shaped sturcture or a circle; (C) three projections forming a big circle. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
CHAPTER 7

COMPARISON OF TWO SILVER STAINING TECHNIQUES FOR DETECTING LIPOPOLYSACCHARIDES IN POLYACRYLAMIDE GELS

7.1 ABSTRACT

The classic silver staining method for detecting bacterial lipopolysaccharides (LPSs) in polyacrylamide gels (C. Tsai and C. E. Frasch, Anal. Biochem. 119:15-119, 1982) was at least 20 times more sensitive than the modified silver staining method (A. Fomsgaard, M. A. Freudenberg, and C. Galanos, J. Clin. Microbiol. 28:2627-2631, 1990) for detecting LPS from a gram-negative bacterium Moraxella osloensis. However, the classic method is only about 3-4 times more sensitive than the modified method for detecting LPSs from Escherichia coli J5, E. coli EH100, E. coli O111:B4 or Salmonella typhimurium. The sensitivity of the modified method for detecting M. osloensis LPS increases about 1-2 times by increasing the periodic acid oxidation time from 20 min to 100 min. The reduction of sensitivity is due to the omission of the initial fixing step (40% ethanol-5% acetic acid, overnight) in the modified method. It is suggested that the
retention of the LPS factions in the gels during fixing and/or oxidation depends on the structure of their lipid A moieties but not the number of the fatty acids in the lipid A moieties.

7.2 INTRODUCTION

Lipopolysaccharides (LPSs) are the important constituents of the outer membranes of gram-negative bacteria as they are the source of O antigen and endotoxin (Galanos et al., 1977; Rietschel et al., 1982). Therefore, LPS plays a major role in the pathogenesis of gram-negative infections and is an important field of study in clinical microbiology (Enright and McKenzie, 1997). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by visualization with silver staining has been used extensively to characterize LPS including the determination of the type of LPS (smooth or rough) (Storm et al., 1991), estimation of their molecular weights (Vaneechoutte et al., 1990; Edebrink et al., 1994), and quantitation of LPS (Gu et al., 1995).

Tsai and Frasch (1982) first reported a highly sensitive classic silver staining method for detecting LPS in polyacrylamide gels. This classic method can detect even less than 5 ng of rough type of LPS. However, Fomsgaard et al. (1990) revealed that the classic method did not stain certain LPS preparations containing a low number of fatty acids which were washed out of the gel during the initial fixing step (40% ethanol-5% acetic acid, overnight). Thus, they developed a modified silver staining method by omitting the fixing step and increasing the LPS periodic acid oxidation (the second step) time from 5 min to 20 min to restore the ability to detect all LPSs.
We purified LPS from *Moraxella osloensis*, a gram-negative bacterium associated with a lethal slug-parasitic nematode, *Phasmarhabditis hermaphrodita* (Tan and Grewal, 2001a, b). The LPS of *M. osloensis* has been found to be an endotoxin that is active against the gray garden slug *Deroceras reticulatum* (Tan and Grewal, 2002). Analysis of different quantities of the purified *M. osloensis* LPS or four commercially available LPSs from other bacteria by SDS-PAGE followed by each of the two methods revealed that the modified method was less sensitive than the classic method.

### 7.3 MATERIALS AND METHODS

**Source of LPS**

*M. osloensis* LPS was purified by classical phenol-water extraction (Westphal and Jann, 1965), with modification as described by Gu et al. (1995), from 3-day pure cultures of *M. osloensis* supplied by MicroBio Ltd., Cambridge, United Kingdom. LPS preparations from *Escherichia coli* J5 (Rc mutant), *E. coli* EH100 (Ra mutant), *E. coli* O111:B4, and *Salmonella typhimurium* were purchased from Sigma Chemical Company, St. Louis, MO.

**SDS-PAGE and silver stain**

The purified or purchased LPS preparations were treated for 5 min at 100°C in 0.05 M Tris-HCl buffer (pH 6.8) containing 2% (wt/vol) SDS, 10% (wt/vol) sucrose and 0.01% bromophenol blue. Ten microliters of each sample was then loaded on a precast Ready Gel® Tris-HCl polyacrylamide gel (86 × 68 × 1.0 mm) containing 4% and 15% acrylamide in the stacking and separating gels, respectively (Bio-Rad Laboratories, Inc.,
Hercules, CA). Electrophoresis was performed at 12 mA in the stacking gel and 25 mA in the separating gel until the bromophenol blue had run about 6.7 cm. LPSs in the gel were then visualized by either the classic method (Tsai and Frasch, 1982) or the modified method (Fomsgaard et al., 1990). In addition, the purified *M. osloensis* LPS in the gel was also visualized by the modified method except that the periodic acid oxidation time was increased from 20 min to 100 min.

### 7.4 RESULTS AND DISCUSSION

The sensitivities of the two methods were compared by using from 50 ng to 5 µg of *M. osloensis* LPS (Figure 7.1). The LPS was revealed to be a rough-type LPS, because only one main band was detected in the gel by both methods. However, 50 or 100 ng of the LPS was not detectable by the modified method whereas the same amount of the LPS gave visible bands by the classic method. Furthermore, the band patterns obtained at 50 ng by the classic method was equivalent to or better than those obtained at 1 µg by the modified method. Therefore, the classic method is at least 20 times more sensitive than the modified method for detecting *M. osloensis* LPS.

The sensitivities of the two methods were also compared by using the same quantities of rough-type LPS from *E. coli J5* or *E. coli EH100* (Figure 7.2). 50 ng of the LPS from *E. coli J5* or *E. coli EH100* was hardly detectable by the modified method whereas the same amount of the LPS gave clear bands by the classic method. Moreover, the band patterns obtained at 50 ng by the classic method was better than those obtained at 100 ng by the modified method. However, the band patterns obtained at 1 µg by the
classic method was less clear than those obtained at 5 µg by the modified method. Thus, the classic method is about 3-4 times more sensitive than the modified method for detecting the rough type LPS from *E. coli* J5 or *E. coli* EH100.

Smooth-type LPS from *E. coli* O111:B4 or *S. typhimurium* were further used to compare the LPS detection sensitivities of the two methods (Figure 7.3). Like the LPS from *M. osloensis*, 50 or 100 ng LPS from *E. coli* O111:B4 was not detectable by the modified method whereas the same amount of the LPS gave visible bands by the classic method. As with the LPS from *E. coli* J5 or *E. coli* EH100, the classic method is about 3-4 times more sensitive than the modified method for detecting the LPS from *E. coli* O111:B4. A similar level (3-4 times) of difference in LPS detecting sensitivity between the two methods was also observed for the LPS from *S. typhimurium*. Although 50 ng or 100 ng of the LPS from *S. typhimurium* was undetectable by the either method, the band patterns obtained at 1 µg or 5 µg by the classic method was much better than those obtained at the same amount of the LPS by the modified method. In brief, the classic method is also about 3-4 times more sensitive than the modified method for detecting the two smooth-type LPS.

The effect of periodic acid oxidation time on the sensitivity of the modified method for detecting *M. osloensis* LPS is shown in Figure 7.4. When the oxidation time was increased from 20 min to 100 min, the band patterns obtained at 1 µg or 5 µg of the LPS became better and clearer. It is estimated that the sensitivity of the modified method for detecting *M. osloensis* LPS increases about 1-2 times with the increase in the oxidation time from 20 min to 100 min. Further, Tsai and Frasch (1982) indicated that the classic method without the periodic acid oxidation of LPS resulted in about five-fold
reduction in sensitivity. Thus, increasing the oxidation time of LPS from 5 min to 20 min in the modified method at least should not reduce LPS detecting sensitivity. Therefore, the reduction of sensitivity is due to the omission of the initial fixing step in the modified method.

It is not fully clear why the detection sensitivities of the two methods differs for *M. osloensis* LPS and other LPSs tested. The four purchased LPSs have different structures of polysaccharide moiety, but had similar level (3-4 times) of difference in LPS detection sensitivity by the two methods. This could be due to the same structure of lipid A moiety being present in the four LPSs (Takayama et al., 1983; Holst et al., 1993). The structure of *M. osloensis* LPS is unknown. Since the structure of lipid A for a given bacterial genus usually exhibits constant characteristics (Rietschel et al., 1980), the structure of lipid A from *M. osloensis* LPS can be forecast from the known lipid A structure for *Moraxella catarrhalis* LPS (Holme et al., 1999). Thus, it is forecast that lipid A from *M. osloensis* LPS contains seven fatty acids, including four 3-hydroxydodecanoic acids, two decanoic acids, and one dodecanoic acid. In contrast, lipid A from the purchased LPSs only contains six fatty acids, including five 3-hydroxytetradecanoic acids and one dodecanoic acid (Takayama et al., 1983; Holst et al., 1993). Fomsgaard et al. (1990) suggested that the retention of LPS fractions in the gels during fixing and/or oxidization may be a property of the number of fatty acids present in their lipid A moiety. However, since lipid A from the purchased LPSs contains a similar number of fatty acids to *M. osloensis* lipid A, the retention of LPS fractions in the gels during fixing and/or oxidation may depend on the structures of their lipid A moieties (e.g. fatty acid pattern or conformation of lipid A) but not the number of the fatty acids in the
lipid A moieties. It is possible that those LPSs containing a low number of fatty acids are fixed weakly or rarely in the gels, whereas *M. osloensis* LPS is fixed much more slowly than other LPSs tested, thus requiring more time (e.g., overnight) to be fixed mostly or completely in the gels.

We conclude that each of the two methods has its individual advantages and disadvantages. The classic method is more sensitive, but time-consuming. Furthermore, it does not detect those LPSs containing a low number of fatty acids. In contrast, the modified method is simpler and faster and detects LPSs that would not be stained by the classic method. However, the present results reveal that the modified method has lower LPS detection sensitivity than the classic method for all LPSs tested, especially that from *M. osloensis*. Therefore, it is suggested that unknown bacterial LPS preparations in the gels be visualized by both methods.

### 7.5 Acknowledgments

This work was supported by a Graduate Research Competitive Grant from the Ohio Agricultural Research and Development Center and by a presidential fellowship of the Ohio State University to L. Tan.

### 7.6 References


Figure 7.1. Comparison of sensitivities of two silver staining methods for detecting *M. osloensis* LPS. (A) Modified method. (B) Classic method. The lanes contain the following amounts of *M. osloensis* LPS: 1, 50 ng; 2, 100 ng; 3, 1 µg; 4, 5 µg. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 7.2. Comparison of sensitivities of two silver staining methods for detecting *E. coli* J5 or *E. coli* EH100 LPS. (A) Modified method. (B) Classic method. Lanes 1 to 4 contain the following amounts of *E. coli* J5 LPS: 1, 50 ng; 2, 100 ng; 3, 1 µg; 4, 5 µg. Lanes 5 to 8 contain the following amounts of *E. coli* EH100 LPS: 5, 50 ng; 6, 100 ng; 7, 1 µg; 8, 5 µg. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 7.3. Comparison of sensitivities of two silver staining methods for detecting \textit{E. coli} O111:B4 or \textit{S. typhimurium} LPS. (A) Modified method. (Note that the background was overstained). (B) Classic method. (Note that a small piece of the gel was lost in the lane 8). Lanes 1 to 4 contain the following amounts of \textit{E. coli} O111:B4 LPS: 1, 50 ng; 2, 100 ng; 3, 1 µg; 4, 5 µg. Lanes 5 to 8 contain the following amounts of \textit{S. typhimurium} LPS: 5, 50 ng; 6, 100 ng; 7, 1 µg; 8, 5 µg. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
Figure 7.4. Effect of 20 (A) and 100 (B) min of periodic acid oxidation on sensitivity of the modified silver staining method for detecting *M. osloensis* LPS. Lane 1 and 2 contain 1 and 5 µg, respectively, *M. osloensis* LPS. The figure was created with Adobe Photoshop 5.5 software (Adobe Systems Inc., 1999).
BIBIOGRAPHY


Wilson, M. J., L. A. Hughes, G. M. Hamacher, L. D. Barahona, and D. M. Glen. 1996. Effects of soil incorporation on the efficacy of the rhabditid nematode,