GENE EXPRESSION IN *MORAXELLA OSLOENSIS*,
*PHOTORHABDUS TEMPERATA* AND *XENORHABDUS KOPPENHOEFERI* DURING HOST INFECTION

DISSERTATION

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ABSTRACT

Bacterium *Moraxella osloensis* is associated with slug-parasitic nematode *Phasmarhabditis hermaphrodita*, and bacteria from genus *Photorhabdus* and *Xenorhabdus* are with entomopathogenic nematode *Heterorhabditis* and *Steinernema*, respectively.

We first determined gene expression of *M. osloensis* in the mollusk host *Deroceras reticulatum* by selective capture of transcribed sequences (SCOTS) technique. Two genes, ubiquinone synthetase (*ubiS*) and acyl-coA synthetase (*acs*) were up-regulated in both *D. reticulatum* and stationary phase *in vitro* cultures. Eleven genes were exclusively expressed in *D. reticulatum* and were thus infection specific. Mutational analysis on genes of protein-disulfide isomerase (*dsbC*) and *ubiS* confirmed that UbiS is important to *M. osloensis* growth and DsbC serves as a virulence factor.

Bacteria *Photorhabdus temperata* and *Xenorhabdus koppenhoeferi*, the symbionts of nematodes *Heterorhabditis bacteriophora* and *Steinernema scarabaei*, respectively, were virulent to three white grub species *Popillia japonica*, *Rhizotrogus majalis*, and *Cyclocephala borealis*. The median lethal dose at 48h post injection
and median lethal time at injection dose of 20 cells per grub showed that *P. temperata* was more virulent than *X. koppenhoferi* to *C. borealis*. However, although *P. temperata* grew faster than *X. koppenhoeferi* both in vitro and in vivo, there were no differences in virulence of two bacteria against *R. majalis* and *P. japonica*. Using SCOTS technique, 30 genes by *P. temperata* and 25 by *X. koppenhoeferi* were identified to be differentially expressed during infection to *R. majalis*. Most genes were unique to either *P. temperata* or *X. koppenhoeferi*, e.g. *ttsL* encoding a type III secretion system was only identified from *P. temperata*, and *lpsE* corresponding to lipopolysaccharide synthesis was unique to *X. koppenhoeferi*. Five nexus genes directly connecting to several other genes were identified as attractive candidates for future studies using the metabolic computational pathway analysis. Compared to the gene expression of *M. osloensis*, several common genes were found to be up-regulated during infection in all three bacteria, including *dsb* gene family and genes encoding outer membrane proteins, fatty acid synthesis proteins, and ubiquinone synthesis proteins.
Dedicated to my parents, wife, and son
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6.2 Functional associations among genes up-regulated in *X. koppenhoeferi* AMK11 during infection to *R. majalis*.................................................................................................150
Slugs (Mollusca: Gastropoda) and white grubs (Coleoptera: Scarabaeidae) are important pests in agriculture and horticulture in the USA. Their phylogeny is shown in Figure 1.1. The grey garden slug, *Deroceras reticulatum*, is regarded as the most serious pest of a wide range of agricultural and horticultural plants in commercial operations, public gardens and home gardens (Hammond *et al.*, 1999; Wilson *et al.*, 1993). Slugs also serve as intermediate hosts for many nematode parasites that pose threats to wildlife and humans (South, 1992). Similarly, white grubs are one of the most serious pests in urban landscapes and pose significant problems in ornamental nurseries and fruit crops (Potter, 1998). Some of the most significant white grub pests are introduced species, such as Japanese beetle (*Popillia japonica*), Oriental beetle (*Anomala orientalis*), European chafer (*Rhizotrogus majalis*) and Asiatic garden beetle (*Maladera castanea*). The Japanese beetle is now the most widespread and destructive insect pest of turf and landscape plants in the eastern United
States, and Oriental beetle, European chafer and Asiatic garden beetle are serious pests predominantly in the northeastern United States (Potter, 1998; Potter & Held, 2002; Vittum et al., 1999).

As one of the widely used control measures, chemical control has become less acceptable, even where it is available and successful. Because the chemical control agents can be detrimental to nontarget organisms, most were restricted or cancelled for turf usage during the 1990s in response to environmental concerns and the 1996 Food Quality Protection Act (Potter & Held, 2002; Zenger & Gibb, 2001). This has increased demand for the development of alternatives.

The slug-parasitic nematodes (SPNs) (Phasmarhabditis) and entomopathogenic nematodes (EPNs) (Steinernema and Heterorhabditis) are important biocontrol agents for pest insects and molluscs (Grewal et al., 2005a), and their phylogeny is presented in Figure 1.1. The SPNs, particularly Phasmarhabditis hermaphrodita, have proven effective against slugs and snails (Wilson et al., 1993). In Europe, these nematodes are now used commercially in agriculture and horticulture for slug control. The EPNs have emerged as excellent biocontrol agents of many soil-dwelling insect pests. They have been used for controlling pests in citrus groves, strawberry plantations, cranberry bogs, production nurseries, green houses and turfgrass.

The symbiotic relationships between nematode and their symbiotic bacteria are less specific in Phasmarhabditis as compared to Steinernema and Heterorhabditis. Phasmarhabditis has been found to be associated with many species of bacteria. Wilson et al. (1995a) recovered over 150 bacterial isolates from infective juveniles (IJs) of P.
hermaphrodita collected from living and dead D. reticulatum, and also from xenic foam chip cultures of these nematodes. However, nematode yield in in vitro cultures and pathogenicity to slugs differ among the different associated bacteria (Wilson et al., 1995a; Wilson et al., 1995b). According to Wilson et al. (1995a), Moraxella osloensis was selected as the preferred associated bacterium for mass-producing P. hermaphrodita in monoxenic culture. Moraxella osloensis is a gram-negative aerobic bacterium that belongs to the family of Moraxellaceae in the gamma subdivision of the purple bacteria. Its phylogenetic position is presented in Figure 1.2. This bacterium is coccal or rod-shaped and pleomorphic. Moraxella osloensis can grow in mineral media with acetate and ammonium salts, and is sensitive to penicillin. It produces oxidases and catalases, but no indoles and pigments. Moraxella osloensis has been isolated from the upper respiratory tract, blood, and cerebrospinal fluid from humans (Bovre, 1984). The bacterium is considered as an opportunistic human pathogen, as it has been found to occasionally cause diseases such as endocarditis (Stryker et al., 1982), osteomyelitis (Sugarman & Clarridge, 1982), central venous catheter infection (Buchman et al., 1993), meningitis (Georgis & Kelly, 1997), pneumonia (Vuori-Holopainen et al., 2001), and endophthalmitis (Berrocal et al., 2002). Phasmarhabditis hermaphrodita has been commercially mass-produced in vitro with the bacterium M. osloensis (Wilson et al., 1995a, b).

Unlike P. hermaphrodita, the symbiotic relationship between species of entomopathogenic nematode Heterorhabditis/Steinernema and their symbiotic bacteria is very specific (Boemare, 2002). These nematodes grow best on their own symbionts, and
Figure 1.1: Tree of eukaryote taxonomy showing the lineage (underlined) of entomopathogenic nematode, slug parasitic nematode, and their host white grubs and slugs. The data were obtained from the taxonomy in National Center for Biotechnology Information, and genome means their genomes are already available or coming soon.
only cells of the specific symbiont are packaged and carried between insect hosts, and are maintained in the intestine of the IJs (Boemare, 2002). These endosymbiotic bacteria belong to the family Enterobacteriaceae. *Heterorhabditis* are associated with *Photorhabdus* and *Steinernema* with *Xenorhabdus* bacteria (Boemare, 2002). The phylogenetic positions for these bacteria are presented in Figure 1.2. Bacteria of the genus, *Photorhabdus* are Gram-negative, usually bioluminescent, and with positive catalase activity. *Photorhabdus* are currently classified as species *luminescens*, *temperata*, and *asymbiotica*. They can be associated with different species of *Heterorhabditis* nematodes. For example, usually *P. luminescens* is associated with *H. bacteriophora*, and *P. temperata* with *H. megidis*. Furthermore, it was recently reported that *P. asymbiotica* is associated with an uncharacterized *Heterorhabditis* species (Gerrard et al., 2006). *P. luminescens* is divided into three subspecies: *P. luminescens luminescens* isolated from *H. bacteriophora* Brecon group, *P. luminescens laumondii* isolated from *H. bacteriophora* HP88, and *P. luminescens akhurstii* isolated from *H. indica* from tropical and sub-tropical regions. Based upon 16S rRNA gene sequence information and metabolic properties, two new subspecies of *P. luminescens*, isolated from *H. bacteriophora*, *P. luminescens kayaii* and *P. luminescens thracensis* have also been proposed (Hazir et al., 2004). There is some confusion for *P. temperata* because this species was also found associated with *H. megidis*, *H. zealandica*, and *H. downesi* (Boemare, 2002). In addition, Hazir (2003) and Burnell and Stock (2000) reported that *P. temperata* was also harbored by *H. bacteriophora*. *Photorhabdus* is closely related to bacteria of the genus *Xenorhabdus*. The genus *Xenorhabdus* is also Gram-negative, but
**Figure 1.2:** Tree of bacterial taxonomy showing the lineage (underlined) of symbiotic bacteria of entomopathogenic and slug parasitic nematode. The data were obtained from the taxonomy in National Center for Biotechnology Information, and genome means their genomes are already available or coming soon.
usually shows negative catalase activity. *Xenorhabdus* species associate with different species of *Steinernema* nematodes, and the diversity of *Xenorhabdus* was recently described by Tailliez et al. (2006).

Slug-parasitic and entomopathogenic nematodes (SPNs and EPNs) have similar parasitic life cycles as demonstrated in Figure 1.3. Naturally, nematode infective juveniles (IJs) carrying bacteria actively seek out and invade suitable hosts through natural body openings. For examples, *P. hermaphrodita* enters into the slug host from the mantle cavity (Wilson *et al.*, 1993; Tan & Grewal, 2001a, b), and *H. bacteriophora* enters into the white grub host through the cuticle, and *Steinernema scarabaei* through the mouth of the white grub (Koppenhöfer *et al.*, 2007). Once the nematode is inside the host, the bacteria are released. For EPNs, the bacteria are released into the insect blood system (Boemare, 2002). For SPNs, it is not reported yet where the bacteria are released. The released bacteria begin to multiply and grow exponentially to a high cell density. The host dies, and the IJs recover into the normal feeding fourth stage. The nematodes feed on symbiotic bacteria completing 1-3 generations in the host cadaver, and as food resources are depleted new IJs are produced which re-associate with the symbiotic bacteria and disperse in search of new hosts.

Several enzymes that are secreted into insect's hemolymph were identified. *Photorhabdus* produces a variety of extracellular enzymes to aid in the degradation of insect tissue. The proteases are primarily zinc metalloproteases of the RTX (Repeats-In-Toxin) family (Bowen *et al.*, 2003; ffrench-Constant *et al.*, 2000), and play a major role in the bioconversion of insect tissue into accessible nutrients for both bacteria and
nematodes (Bowen et al., 2003; Daborn et al., 2001) and in the inhibition of antibacterial factors secreted by the insect (Cabral et al., 2004). Lipases (Wang & Dowds, 1993) and hemolysins (Brillard et al., 2001; Brillard et al., 2002) are hypothesized to function in a similar biodegradation role.

Besides biodegradation enzymes, the bacteria also produce antibiotics to prevent scavengers (other nematodes, insects, bacteria, fungi, etc.) from invading the insect carcass (Akhurst, 1982). Several antimicrobial compounds have been identified, including a red pigment that is an anthraquinone derivative (Li et al., 1995) and a hydroxystilbene with strong antifungal activity (Richardson et al., 1988). A novel catechol siderophore, "photobactin", was identified that is structurally related to the vibriobactins (Ciche et al., 2003). Purified photobactin has detectible antibiotic activity against both Gram-positive and Gram-negative bacteria. A carbapenem-like antibiotic (a Plactam broad-spectrum antibiotic) and the gene cluster involved in its biosynthesis (cpmA to cpmH) have been identified in P. luminescens strain TTO1 (Derzelle et al., 2002). Photorhabdus luminescens W14 produces bacteriocins (termed "lumicins") that have both DNase and RNase activity that kill competing Photorhabdus strains (Sharma et al., 2002). To deter non-microbial pests, Photorhabdus produces an anti-deterrent factor (Zhou et al., 2002) and nematicidal metabolites (Hu et al., 1999). All of these compounds function to protect the nematode-bacterial partners within the insect carcass from scavengers.

Further, several bacterial genes have been identified to be essential for the specific relationship between entomopathogenic nematodes and the symbiotic bacteria.
Figure 1.3: The infective life cycles of slug parasitic and entomopathogenic nematodes.
The *Photorhabdus* mutant in *ngrA* encoding phosphopantetheinyl transferase failed to support nematode growth and reproduction (Ciche *et al.*, 2003). As the *Photorhabdus* mutant in *exbD*, a component of the energy-transducing system, grew poorly within the insect and were also unable to support nematode growth and development *in vitro* (Watson *et al.*, 2005), they suggested that iron may play a key role in the nutritional exchange between the bacteria and nematode: the bacteria probably produce siderophores to scavenge iron from the insect, while the nematodes feed on *Photorhabdus* to satisfy their own iron requirements. The *Xenorhabdus nematophila* mutant in *lrp* was deficient in initiating colonization and growth within its nematode host, and nematodes reared on *lrp* mutant plate exhibit decreased overall numbers of nematode progeny (Cowles *et al.*, 2007). The *pbgPE* operon from *Photorhabdus* is required for bacterial colonization in *H. bacteriophora* IJs.

The pathogenicity of the nematode-bacterium complex to the host depends mainly on the symbiotic bacteria. Tan and Grewal (2001a) discovered that aged cultures of *M. osloensis* were pathogenic to the slug *D. reticulatum* after injection into the shell cavity or the hemocoel, and pathogenicity of *P. hermaphrodita* depended on the number of viable *M. osloensis* carried by the IJs. They 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 (2002) further discovered that purified lipopolysaccharide (LPS) from *M. osloensis* cultures was toxic to the slugs when injected into the shell cavity, thus demonstrating that *M. osloensis* LPS is an active endotoxin. Like *M. osloensis* to slugs, EPNs-associated *Photorhabdus* bacteria are also
toxic to the insect host when injected into the hymolymph (Clarke & Dowds, 1995; Daborn et al., 2001; Watson et al., 2005). *Photorhabdus* pathogenicity is very tightly correlated with its growth rate (Clarke & Dowds, 1995; Daborn et al., 2001; Watson et al., 2005). In the case of *Xenorhabdus*, previous studies showed that *X. nematophila* and *Xenorhabdus bovienii* were highly virulent to *P. japonica* (Yeh & Alm, 1992) and *Hoplia philanthus* (Ansari et al., 2003), respectively, but *X. poinarii* was not virulent to either grub species. Like *M. osloensis*, *Xenorhabdus* spp also produce endotoxins, and *X. nematophilus* endotoxins are LPS components of the cell wall that are toxic to the hemocytes of *G. mellonella* larvae (Dunphy & Webster, 1988). Unlike *M. osloensis*, exotoxin activity has been discovered from the bacteria *P. luminescens*, *X. nematophilus* and *Xenorhabdus bovienii* by injecting the bacterial supernatant into insect hosts (Georgis & Kelly, 1997). Bowen and Ensign (1998) purified a high-molecular-weight extracellular insecticidal protein complex from *P. luminescens*. The purified toxin complex (Tc) contained no protease, phospholipase, or hemolytic activities and only a trace of lipase activity. Tc was found to be effective in nanogram concentrations against insects in four orders (Bowen & Ensign, 1998). Genes, *tca*, *tcb*, *tcc*, and *tcd*, encoding the Tc toxins from *P. luminescens* have been cloned (Bowen & Ensign, 1998). Both the *tca* and *tcb* encode complexes with high oral toxicity to *Manduca sexta* (Bowen et al., 1998). Later, similar Tc gene sequences have been found in *X. nematophilus* (ffrench-Constant & Bowen, 2000). A functional type III secretion system has also been identified in *Photorhabdus*, and this type III secretion system has been shown to secrete LopT, a homologue of YopT from *Yersinia pestis* (Brugirard-Ricaud et al., 2004;
Brugirard-Ricaud et al., 2005). It was recently shown that the mutation of phoP, a two component system, rendered *P. luminescens* avirulent to insect larvae (Derzelle et al., 2004). However, very little is known about how toxin production is regulated, although there is some evidence that *Photorhabdus* bacteria express *tca* and *tcd* during exponential growth in the insect larvae (Daborn et al., 2001).

In the field, the EPNs can be effective as biological insecticides for controlling white grubs in turf (Grewal et al., 2005a) or potted nursery stocks (Wright et al., 1988). However, the use of this bacteria-nematodes complex against grubs can be limited due to the differences in the susceptibility of white grub species to EPNs. As geographic ranges of different white grub species overlap and susceptible species often occur sympatrically (Vittum et al., 1999), it is essential to have a nematode strain that provides acceptable control of all these grub species in a particular area in order to compete with chemical insecticides.

Some newly identified nematodes have improved efficacy against grub control (Koppenhöfer et al., 2000). Grewal et al. (2002; 2005b) revealed that the GPS11 strain of *H. bacteriophora* has the highest virulence against *P. japonica*, *A. orientalis*, and *Cyclocephala borealis*, but it is relatively ineffective against *R. majalis*. Commercially, the GPS11 strain has been mass-produced *in vitro* for the control of *P. japonica*, *C. borealis* and *A. orientalis* (Cyrille Verdum, Becker Underwood, Ames, Iowa). Recently it has been reported that the nematode *Steinernema scarabaei* with its associated bacteria *Xenorhabdus koppenhoeferi* was highly virulent against *R. majalis* (Cappaert & Koppenhöfer, 2003; Koppenhöfer et al., 2004). However, the fact that *S. scarabaei* can
not be mass-produced *in-vitro* was a biggest disappointment. Even if the mass-production becomes possible with additional research, the process will still remain costly due to the extremely large size of IJs (Grewal *et al.*, 2005b). As GPS11 strain of *H. bacteriophora* can be efficiently mass-produced and has shown excellent field efficacy against *P. japonica, C. borealis, C. hirta,* and *A. orientalis,* its virulence against *R. majalis* must be improved.

The availability of the *P. luminescens* TTO1 genome sequence has been a big advantage for studying EPNs and SPNs. Several pathogenicity islands have been identified in the genome of TTO1 (Duchaud *et al.*, 2003; Waterfield *et al.*, 2002). For example, one island encodes Mcf (makes caterpillars floppy) toxins which were reported to induce apoptosis in eukaryotic cells (Daborn *et al.*, 2002; Waterfield *et al.*, 2001). The genome sequences of *X. nematophila* and *X. bovienii* will be in the public domain soon. This will provide new insights into the pathogenesis of these nematode symbiotic bacteria.

When facing a big bacterial genome with many open reading frames, the question is how we can decide which genes to be focused on? As the interaction between a host and bacterium requires the coordinated activity of many bacterial gene products in response to the host, *in vivo* differentially expressed genes provide excellent insight into what bacteria are doing during the infection process and how bacteria selectively employ their genome during interaction with the host.

The current study has focused on the topics concerning host–bacteria interactions, covering three nematode associated bacteria: *M. osloensis, Xenorhabdus koppenhoeferi,* and *Photorhabdus temperata.* A comprehensive analysis of genes involved in the
infection processes of these three bacteria was performed. Although strategies or the mechanisms they used to infect the hosts may vary greatly, it is still important to compare them with each other since they are all pathogenic nematode associated bacteria.

One of the important strategies for studying host-bacteria interactions is the identification of in vivo expressed genes during infection of the host. Prior to the start of this study, because genetic information and genetic studies of Moraxella were limited, looking for a suitable approach was very important. The techniques for in vivo gene expression analysis include in vivo expression technology (IVET) (Mahan et al., 1993), signature-tagged mutagenesis (STM) (Hensel et al., 1995), and differential fluorescence induction (DFI) (Valdivia & Falkow, 1997). Recently, the selective capture of transcribed sequences (SCOTS) technique has been developed to study bacterial gene expression in macrophages (Graham & Clark-Curtiss, 1999). This technique has been used to identify bacterial virulence factors, and was demonstrated to have sufficient sensitivity for the identification of expressed genes in specific tissues of infected animals (Baltes & Gerlach, 2004; Dozois et al., 2003). Since the SCOTS technique can differentiate host and bacterial cDNAs, and does not require genetic information of the pathogen, we used SCOTS technique in the current study to determine gene expression of SPN and EPN symbiotic bacteria in their hosts during infection. Therefore, this study begins from a mini review on the methodology of in vivo gene expression (Chapter 2). SCOTS technique was applied to profile M. osloensis genes that are up-regulated during infection to a slug (Chapter 3). Are these up-regulated genes comparative to that in EPN associated bacteria? To answer this question, the symbiotic bacteria Photobacterium and
Xenorhabdus from two important EPNs (*H. bacteriophora* GPS11 and *S. scarabaei*) were also covered in this project, including isolation and identification of the symbiotic bacteria from two important EPNs (Chapter 4), mechanisms of different susceptibility of white grubs to EPNs: due to the symbiotic bacteria or nematode (Chapter 5), and profiling the *in vivo* gene expression during the grub infection (Chapter 6), as well as suggestions for future studies.

**REFERENCES**


CHAPTER 2

A REVIEW OF THE USE OF SELECTIVE CAPTURE OF TRANSCRIBED SEQUENCES TECHNIQUE FOR STUDYING BACTERIAL INFECTIONS

2.1 SUMMARY

Bacterial infection is a complex process. The identification of bacterial genes expressed in vivo can aid in the understanding of the molecular mechanisms of pathogenesis. In the past 15 years, several techniques have been developed to investigate bacterial gene expression within their hosts. The most commonly used techniques include in-vivo expression technology, signature-tagged mutagenesis, differential fluorescence induction, and cDNA microarrays. However, the limitations of these techniques in analyzing bacterial in vivo gene expression stimulated the development of alternative tools. Selective capture of transcribed sequences (SCOTS) is a recently developed method specifically for analyzing bacterial in vivo gene expression. Due to its
huge advantage of easily separating the bacterial mRNA transcripts from the host, this technique has become an elegant tool for virulence gene discovery in bacterial pathogens. In this review, we summarize the advances in the development and use of SCOTS technique, including its current and potential application to bacterial gene expression induced under a variety of in vivo conditions.

2.2 INTRODUCTION

The interactions between bacterial pathogens and their hosts require the activity of many bacterial gene products. Profiles of bacterial genes that are expressed during infection may provide valuable information. In the past, much of the knowledge about the bacterial virulence determinants came from experiments with bacteria grown in vitro by simulating the host environment with specific culture conditions such as pH, temperature, and iron levels. Although in vitro assays have been useful, it is obvious that they cannot accurately reproduce all aspects of the bacterial pathogenesis. Today, lots of sequenced bacterial genomes, with many more currently being sequenced, provide tremendous new opportunities for research of bacterial pathogenesis. Several techniques have been developed to identify bacterial genes expressed specifically in host during infection. Moreover, the ability to investigate bacterial in vivo gene expression when genome sequences are not available has become possible. These methods are either based on protein or gene level analysis. The protein-based methods are performed to determine the gene product under different bacterial growth conditions, and the gene-based methods are applied to analyze the transcripts under different bacterial growth
conditions. Currently, the protein-based methods include two-dimensional gel electrophoresis (2-D gel), multidimensional protein identification technology (MudPIT), and *in vivo* induced antigen technology (IVIAT) (Deb *et al*., 2002). The gene-based methods are either mutagenesis based or PCR-hybridization based. Mutagenesis based methods include *in vivo* expression technology (IVET) (Mahan *et al*., 1993), differential fluorescence induction (DFI) (Valdivia & Falkow, 1996), signature-tagged mutagenesis (STM) (Hensel *et al*., 1995), and genomic analysis and mapping by *in vitro* transposition (GAMBIT) (Judson & Mekalanos, 2000). PCR-hybridization based methods include differential-display reverse transcription-PCR (DDRT-PCR) (Liang & Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu *et al*., 1995), suppressive subtractive hybridization (SSH) (Diatchenko *et al*., 1996), real-time PCR (RT-PCR) (Heid *et al*., 1996), cDNA microarray (DeRisi *et al*., 1996), and selective capture of transcribed sequences (SCOTS) (Graham & Clark-Curtiss, 1999b). One of these methods, RT-PCR is based on detection of a fluorescent signal produced proportionally during PCR amplification, and it provides an accurate method for determination of levels of specific DNA and RNA sequences in tissue samples. The significant limitation is that RT-PCR requires knowledge of the gene to design the primers, and cannot be used as a gene expression scanning tool (Shelburne & Musser, 2004), thus it will not be discussed in this review. However, RT-PCR has qualities to bridge other methods, allowing accurate gene expression measurements. In this review, we provide a brief discussion on most techniques facilitating identification of differentially expressed bacterial genes or differentially produced proteins within the host. The purpose of this review is to share
the information about the techniques that have been developed, and to discuss the proper conditions that each technique can be used in, especially with regards to the SCOTS applications.

### 2.3 PROTEIN BASED METHODS

Protein based methods refer to analysis of bacterial gene products produced *in vivo*, and they have the potential to directly measure mRNA transcript levels. There are three main protein based methods for analyzing bacterial gene products *in vivo*. One is two-dimensional electrophoresis (2-D gel), one is multidimensional protein identification technology (MudPIT), and another is *in vivo* induced antigen technology (IVIAT).

2-D gel relies on the separation of whole proteins by gel electrophoresis from two dimensions. These proteins can then subsequently be analyzed by mass spectrometry. In the first dimension, isoelectric focusing separates the proteins on the basis of charge. In the second dimension the proteins are resolved according to molecular weight using SDS-PAGE. The proteins thus separated from the mix of the sample are then visualized by staining with Coomassie Blue dye or silver staining. Individual proteins are seen as spots with specific 2D coordinates (charge and size) on the gel. MudPIT relies on the separation of proteolytic peptides by liquid chromatography and subsequent identification by electrospray ionization-tandem mass spectrometry. Technically, 2-D gel often excludes large and hydrophobic proteins, and the MudPIT approach overcomes this limitation. However, 2-D gel provides a visual reference to compare gene products, posttranslational modifications, and protein cleavage events, which would not be evident
by using MUDPIT. In addition, unlike 2-D gel, MUDPIT does not yield quantitative information (Gygi et al., 1999). Thus, in order to overcome the problems limiting the coverage of proteomic analysis, both techniques should be complementarily used (Schmidt et al., 2004). Several studies have been reported to analyze bacterial protein production during growth in-vitro under conditions that mimic some aspects of infection. Theoretically, in order to identify in vivo induced gene products, 2-D gel can be performed to compare proteins present in vitro and in vivo, but up to now, no studies of global protein production analysis of a bacterial pathogen within its natural host or an animal model have been published. This is because of the technical hurdles associated with separating bacteria from the host tissues and obtaining enough material to perform serial statistical analysis. Thus, the potential of this technique for bacterial in vivo gene expression analysis is limited. An improved strategy would be pre-fractionation such as sub-cellular fractionation and selective removal of dominant protein components (Hamdan & Righetti, 2003; Zuo et al., 2001). However, there is no report on the influence of pre-fractionation to the protein separation qualitatively and quantitatively.

In vivo induced antigen technology (IVIAT) has been developed to identify proteins expressed by pathogenic bacteria during an actual infectious process. IVIAT uses pooled sera from infected animals to probe genes expressed specifically in vivo. The pooled sera from animal or specific tissue are repeatedly absorbed with in vitro grown cells and lysates of cells (antigen), respectively, leaving antibodies against antigens expressed only in vivo. Genomic expression libraries of pathogen are generated in Escherichia coli and clones are probed with the absorbed sera to identify the specifically
in vivo expressed factors. This method overcomes limitations of animal models, allowing direct identification of microbial proteins expressed during human infection (Handfield et al., 2000; Handfield et al., 2002; Hang et al., 2003). However, because this technique is based on the immune reactions, its application to invertebrates is limited. Another limitation is the immune cross-reactions due to the simultaneous exposure to a variety of pathogens (Rollins et al., 2005).

2.4 MUTAGENESIS BASED METHODS

In vivo expression technology (IVET) is a promoter-trap technology that has been developed to select bacterial genes specifically induced during infection. This technique rests on positive selection to identify genes turned on during in vivo growth. Because IVET vectors contain a random fragment of the chromosome of the bacterial pathogen and a promoter-less gene encoding a selective marker that is required for survival, random integration of the IVET vector into the pathogen chromosome creates a pool of recombinant pathogens. Pooled clones are then inoculated into the host. Only those bacteria that contain the selective marker fused to a gene that is up-regulated in the host are able to survive. After a suitable infection period, bacteria that express the marker are isolated to screen the in vivo expressed genes. There are several variations of IVET, and each relies on the generation of transcriptional fusions of genomic sequences to a reporter gene such as purA or antibiotic gene (Angelichio & Camilli, 2002), promoterless resolvase gene such as tnpR from Tn (Veal-Carr & Stibitz, 2005). The major technical problems limiting the use of this technique include the suitable animal model, and the
availability of transformation and recombination mechanisms in the organisms of interest (Angelichio & Camilli, 2002).

Differential fluorescence induction (DFI) is another promoter-trap based method. The differentially expressed genes are identified by a genetic selection via fluorescence-enhanced green fluorescent protein (GFP) and a fluorescence activated cell sorter (FACS). The bacteria are separated by FACS in response to the expression, or lack of expression along with the fluorescent marker. Like IVET, a common major disadvantage of DFI strategy is that the animal model is required, which may not be adequately replicated by bacterial pathogen in the host (Rollins et al., 2005).

Signature-tagged mutagenesis (STM) is a negative selection technique in which a pool of sequence-tagged mutant bacteria is administered to an animal in an appropriate model. Mutations represented in the initial inoculum but not recovered from the host are identified as virulence factors for essential colonization in host (Mecsas, 2002). However, this technique is limited by the need for an adequate model that facilitates recovery of bacteria from an infected host. Mutants that are slow-growing, inviable, contain mutations in genes encoding redundant functions, or that can be complemented in a mixed population may be underrepresented (Lehoux & Levesque, 2000; Mecsas, 2002; Shea et al., 2000). For detailed information, readers can consult recent published reviews (Autret & Charbit, 2005; Saenz & Dehio, 2005).

Genomic analysis and mapping by in vitro transposition (GAMBIT) is introduced to identify the essential genes that are required for bacterial pathogen growth in-vitro or in vivo. A specific region of the chromosome is amplified by extended-length PCR, and
the product is subjected to *in vitro* transposon mutagenesis. The resultant pool of mutagenized DNA is then transformed into bacteria, which are then grown under selective conditions (e.g. on defined medium or in an animal). PCR is then performed on the postselection pool using a transposon-specific primer and a primer to a known location on the chromosome. Subsequent analysis of the PCR products allows determination of which genes in that region of the chromosome are required for survival under those selective conditions. Because such genes are expressed under all conditions, they will not be identified by other methods such as IVET or STM unless they were expressed at extremely low levels *in-vitro* relative to *in vivo* (Lehoux *et al.*, 2001; Lowe *et al.*, 1998). Like STM, the use of GAMBIT in animal models constitutes a negative selection in which certain mutants are eliminated by selection in the animal. These mutants are recognized by the loss of PCR products corresponding to insertions in the *in vivo* essential genes that are represented in the preinfection inoculum. The major disadvantage is the necessity to design quite a large number of PCR primers to apply GAMBIT to entire genomes, and it can only be applied to naturally competent bacterial cells. Therefore, the development of efficient DNA transformation methods should enable the adaptation of this system for the analysis of bacteria that are not naturally competent (Akerley *et al.*, 1998).

### 2.5 PCR-HYBRIDIZATION BASED METHODS

Differential-display reverse transcription-PCR (DDRT-PCR) technique allows extensive analysis of gene expression among several cell populations. The DDRT-PCR
procedure consists of two major steps: Reverse transcription (RT) of mRNAs isolated from different cell populations with a set of degenerate, anchored oligo-dT primers to generate cDNA pools; and PCR amplification of random partial sequences from the cDNA pools with the original anchored dT primer and an upstream arbitrary primer. The differential gene expression was obtained by comparing the PCR products among the different cell populations. Studies with bacterial pathogens mainly include comparison of gene expression between virulent and nonvirulent strains (Rindi et al., 1999; Rivera-Marrero et al., 1998; Sturtevant, 2000). This technique may provide an unbiased method to compare mRNA pools from two or more samples, but several limitations including difficulty to obtain good quality mRNA and isolation of PCR products, the large number of false-positive results, and difficulty to confirm differential expression, limit its advance in bacterial gene expression studies (Sturtevant, 2000).

Suppressive subtractive hybridization (SSH) is based on the strategy of subtractive hybridization to identify the differentially expressed genes. cDNAs are synthesized from mRNA isolated from the two populations of interest. The reference cDNA is called the subtractor and the cDNA where the differentially expressed transcripts are to be found is called the target. The target cDNA population is constructed by random priming. Common cDNAs between target and subtractor populations anneal in a hybridization reaction in the presence of excess subtractor cDNA. After removing the subtractor cDNA, PCR amplification is performed with primers complementary to each of the random primed sequences, so only the differentially expressed cDNA was amplified. Tennant et al (2005) used SSH to identify potential virulence genes of
pathogenic strains of *Y. enterocolitica* by determine genetic differences between virulent and avirulent strains. The products of the identified toxin gene complex were found to contribute to the virulence of some strains of *Y. enterocolitica* due to its facilitating the bacterial persistence *in-vivo*. However, the major disadvantage of this technique is also the separating the bacterial mRNA from the host mRNA, and more mRNA is required (Hamdan & Righetti, 2003).

Serial analysis of gene expression (SAGE) is basically a rapid method to obtain short sequences of all expressed genes in a given situation (Velculescu et al. 1995). Advantages of this method include high throughput and the ability to compare SAGE data from numerous populations (Velculescu et al. 1995). However, preparation of SAGE libraries can be technically difficult, and bioinformatic tools are required for analysis of data (Sturtevant, 2000). From 2003, there are no report on the studies of bacterial pathogen and host interaction by using SAGE technique. For more detail, a superb review paper on SAGE with updated information can be referred (Matsumura et al. 2005).

cDNA microarrays are used to determine the difference in mRNA levels among bacterial strains grown *in vitro* and *in vivo*. mRNAs were isolated from two cell samples, and a separate dye for each of the two cDNA pools being compared is incorporated into the reverse transcription step. After allowing for hybridization of the cDNA to the microarray, the differential fluorescence of the two cDNA pools is measured and the ratio is determined for each gene on the microarray that reflect the difference in mRNA level. Theoretically, cDNA microarrays offer the promise of accurate gene expression
measurements for every gene in a genome. However, this huge potential has not been realized because of the substantial technical problems. The main problems include the lack of a large set of clones of known genes to array, the low numbers of bacteria in living tissues during infection, and difficulty in purifying the bacteria from the eukaryotic tissue. Therefore, cDNA microarray can currently only be applied to bacterial infections that lead to high titers in host tissues (Boyce et al., 2004; Hinton et al., 2004; Jansen & Yu, 2006). Although a new method named differential expression analysis using a custom-amplified library (DECAL) has been developed to work along with cDNA microarray to overcome the limitation of quantity (Alland et al., 2002; Narasimhan et al., 2003; Shelburne & Musser, 2004), the potential use of DECAL suffers from having limited range for detection of mRNA and cannot provide direct differential quantitation (Shelburne & Musser, 2004).

The development of several elegant techniques in recent years to study in vivo gene expression enables in-depth exploration of bacterial pathogen and host interactions. However, the common limitations of these techniques include the isolation of abundant high quality mRNA, separating bacterial mRNA from host mRNA, the requirement of proper animal models, and the requirement of bacterial prior genetic information. In addition, for IVET and STM techniques, the different growth rates of individual transformant within the pool require a termination of the experiment within hours after infection, and they are not applicable to the identification of genes involved in the later stages of the infection (Baltes & Gerlach, 2004). An improved approach, the selective capture of transcribed sequences (SCOTS) overcomes most of these limitations noted
above. Unlike other methods with physical limitation of separating the bacterial mRNAs from host mRNAs, SCOTS technique displayed in Fig. 2.1 is a PCR-select technique that selective capture occurs by subtractive hybridization and selective amplification of differentially expressed genes. Subtraction is a revolutionary method for finding differentially expressed genes that expressed in one condition but reduced or absent in another (Diatchenko et al., 1996; Gurskaya et al., 1996)

2.6 SCOTS TECHNIQUE

SCOTS is a positive selection technique which is used to identify bacterial genes expressed in the host during infection. This technique was originally developed by Graham and Clark-Curtiss in 1999 (Graham & Clark-Curtiss, 1999a). SCOTS allows selective capture of a great diversity of bacterial cDNAs that are up-regulated or over-expressed by bacterial pathogens during infection. Briefly, the total RNA is isolated either from bacteria grown in vitro or bacteria-infected host tissues and converted to cDNA by using random primers with an adapter overhang. The bacterial cDNA is captured by hybridization with biotinylated-sonicated bacterial chromosomal DNA that has been prehybridized with bacterial rDNA sequences. Prehybridization with rDNA allows an effective capture of the cDNA molecules representing mRNA transcripts. The cDNA-chromosomal DNA hybrids are captured by streptavidin-coated magnetic beads. The captured cDNAs are then eluted and PCR amplified. The amplified cDNAs are denatured, and again hybridized to genomic DNA-rDNA mixture for two successive rounds of SCOTS. The finally captured in vivo cDNAs are subtracted by in vitro cDNA
Figure 2.1: Flow chart of selective capture of transcribed sequences (SCOTS). Total RNA is isolated from in vitro and in vivo bacterial cells. Primers with a defined sequence at the 5’ end and random nonamers at the 3’ end are used for both first and second strand cDNA synthesis. Sonicated biotinylated bacterial genomic DNA is preblocked with bacterial rRNA operon in order to effectively capture the cDNA molecules representing mRNA transcripts. Prepared cDNAs are added to the DNA-rRNA prehybridized mixture to hybridize for 24h. Streptavidin paramagnetic particles are used to capture the bacterial cDNA and genomic DNA hybrids. The captured hybrids are PCR amplified with the defined primer. The bacterial cDNAs representing up-regulated mRNA transcripts in vivo can be enriched by subtracting SCOTS captured in vitro cDNAs from SCOTS captured in vivo cDNAs.
mixtures. The subtraction is performed by hybridizing selectively captured \textit{in vivo}
cDNA mixtures with rDNA-chromosomal DNA mixtures that has been preblocked with
selectively captured \textit{in vitro} cDNA mixtures. After three rounds of subtraction, bacterial
cDNAs are cloned into a cloning vector. Each individual cloned inserts are screened by
southern blot hybridization with probes made from selectively captured \textit{in vitro} or \textit{in vivo}
cDNA mixtures. Clones that only hybridized to the probe made from selectively
captured \textit{in vivo} cDNA mixtures are chosen for sequence analysis. Sequence analysis can
be carried out using BLAST algorithms in GenBank NCBI.

SCOTS technique was originally developed to identify genes expressed by
\textit{Mycobacterium tuberculosis} growing in macrophages, and was subsequently used for the
isolation of macrophage-activated \textit{M. avium} (Hou \textit{et al.}, 2002) and \textit{Salmonella enterica}
serovar Typhi genes (Daigle \textit{et al.}, 2001). SCOTS has been successfully used for
identification of gene expression in a diversity of bacterial pathogens (Table 2.1). \textit{In vitro}
work with \textit{Listeria monocytogenes}, a food-borne bacterial pathogen that is able to
grow at refrigeration temperatures, 24 different cDNA clones were identified by SCOTS.
SCOTS has also been used successfully to identify the bacterial gene expression in
specific tissues. \textit{Helicobacter pylori} inhabit a highly restricted ecological niche in the
human gastric mucosa. The gene expression of \textit{H. pylori} in the context of persistent
infection remains largely uncharacterized before applying SCOTS analysis (Graham \textit{et al.}, 2002). The majority of SCOTS identified cDNAs are corresponding to the factors
unique to \textit{H. pylori} that are potentially produced in response to interactions with
mammalian gastric mucosa. The pathogenic \textit{Escherichia coli} strains cause a variety of
<table>
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<tr>
<th>Bacteria species</th>
<th>Pathogen source</th>
<th>Reference</th>
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<tr>
<td><em>Mycobacterium tuberculosis</em></td>
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<td>Graham and Clark-Curtiss, 1999</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>Human macrophages</td>
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<tr>
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<td>Human macrophages</td>
<td>Daigle et al., 2001</td>
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<td>Human macrophages</td>
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<tr>
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<td>Human macrophages</td>
<td>Faucher et al., 2006</td>
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Table 2.1: SCOTS technique used in bacterial pathogen *in vivo* gene expression studies

diseases in different host species. Avian pathogenic *E. coli* strain 7122 has been used in a chicken infection model to identify bacterial genes that are expressed in infected tissues (Dozois et al., 2003a). Using SCOTS technique, pathogen-specific cDNAs corresponding to putative adhesins, lipopolysaccharide core synthesis, and iron-responsive etc. have been identified in *E. coli* strain 7122. *Actinobacillus pleuropneumoniae* is the porcine respiratory tract pathogen that presents a major problem to the swine industry due to its ability to persist in the host, surviving in tonsils as well as in sequestered necrotic lung tissue, which leads to the occurrence of subclinically infected carrier animals. Genes expressed by *A. pleuropneumoniae* in necrotic porcine lung tissue have been identified by SCOTS (Baltes & Gerlach, 2004). Almost half of the
identified genes have been previously reported to be associated with \textit{in vivo} expression of virulence in \textit{A. pleuropneumoniae} or in other organisms, which strongly suggests that SCOTS analysis is a suitable tool for the study of gene expression in infected porcine lung tissue.

SCOTS derived cDNA probe mixtures from the specific growth condition can be further used to determine the differential gene expression during the bacterial infection process (Daigle \textit{et al.}, 2002). The \textit{trcRS} two-component system of \textit{M. tuberculosis} is comprised of the TrcS histidine kinase and the TrcR response regulator, which is homologous to the OmpR class of DNA binding response regulators. Analysis of the \textit{trcR} and \textit{trcS} genes using various SCOTS probes confirmed that these genes are expressed in broth-grown cultures and after 18 h of \textit{M. tuberculosis} growth in cultured human primary macrophages (Haydel \textit{et al.}, 2002). Related study on the gene \textit{rv1057} by using SCOTS derived probes has shown that the \textit{rv1057} is expressed during early \textit{M. tuberculosis} growth in human macrophages, and its expression profile correlates with a gene that negatively regulated by TrcR. Therefore, SCOTS technique can be used as an evaluation tool for the analysis of intracellular gene expression (Haydel & Clark-Curtiss, 2006).

Moreover, SCOTS can be used to determine the expression of any gene of interest by SCOTS derived cDNA probe mixtures from the desired growth condition. The global regulatory mechanisms of \textit{M. tuberculosis} that coordinate environmental or intracellular gene expression have been studied using SCOTS technique. Expression of the 17 response regulator genes and the two orphan histidine kinase genes during growth of \textit{M. tuberculosis}...
*tuberculosis* in human peripheral blood monocyte-derived macrophages has been analyzed using cDNA probe mixtures prepared by the SCOTS technique. Based on expression profile, the regulatory genes have been assigned to three categories: constitutively expressed during growth in macrophages, differentially expressed during growth in macrophages, and no detectable expression during growth in macrophages (Haydel & Clark-Curtiss, 2004).

SCOTS technique has the great potential to determine the differential transcription in host cells or tissues by comparative blocking between different strains that belong to the same or similar species with high overall DNA homology but with different degrees of virulence or host specificities (Dozois *et al.*, 2003b). Identification of pathogen-specific and conserved bacterial genes that are expressed *in vivo* will provide further insight into the mechanisms by which bacteria colonize host tissues, cope with, or circumvent host defenses and adjust to the nutrient limitations and other stresses that occur in different host environments. In addition, SCOTS has the potential to identify differences in bacterial transcripts at different localizations such as tonsils and unaltered lung tissue as well as in even later stages of infection.

Besides being used in a stand-alone fashion, results generated with SCOTS have been used in conjunction with cDNA microarray to validate the identified cDNAs data and to determine global gene expression by a pathogen during infection (Faucher *et al.*, 2006; Graham *et al.*, 2002). Bacterial cDNAs obtained by SCOTS from tissues hybridized to arrayed DNA fragments representing approximately 70% of open reading frames (ORFs) in the *H. pylori* genome (Graham *et al.*, 2002). Faucher et al. (2006)
studied gene expression of *Salmonella typhi* using cDNA microarray with SCOTS-derived probes. The SCOTS-cDNA mixture displayed an expected expression profile of *S. typhi* virulence genes from infected macrophages. Using SCOTS and microarray analysis, the transcriptome of intracellular bacteria can be obtained without altering the existing infection model.

### 2.7 SCOTS VERSUS OTHER METHODS

A comparison among various *in vivo* gene expression techniques is presented in Table 2.2. The SCOTS technique can be applied to any bacterial pathogen, with no requirement for specialized genetic techniques and species-specific cloning vectors. Unlike IVET or DFI strategy, SCOTS identifies relevant genes, rather than promoter regions. SCOTS also has the advantage to detect *in vivo* gene expression from the small numbers of bacterial cells obtained in samples from living tissues in natural states, including human biopsies, an application for which other methods are currently not available. Because of this advantage, the combination of SCOTS and cDNA microarrays will be an effective way to determine the bacterial gene expression and can be used to investigate transcriptional profiles of other bacterial pathogens without the need to recover many nanograms of bacterial mRNA from host and without increasing the multiplicity of infection beyond what is seen in nature. The application of SCOTS and cDNA microarrays on tissue biopsies from infected patients, as demonstrated with *H. pylori* in human biopsy samples (Graham *et al.*, 2002), has the potential to further elucidate the bacterial genes expressed during infection of the human host. However, the
<table>
<thead>
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</table>

**Table 2.2:** Comparison among the techniques used in bacterial *in vivo* gene expression

SCOTS technique alone does have some limitation. For example, it is not a quantitative method for gene expression studies. In addition, a common feature for all of the above techniques is that they all need post-process to evaluate and validate the identified *in vivo* expressed genes to be virulence related genes.

### 2.8 CONCLUSIONS

New methods have been developed to simplify the analysis of bacterial genes actively transcribed in the host, and such studies will transform our understanding of the molecular mechanisms of pathogenesis. It must be emphasized that most of these techniques only identify potentially differentially expressed genes and that confirmation of the expression and the role of each gene in bacterial pathogenesis is still necessary. As
noted in this review, none of the currently available techniques is perfect. The best strategy right now regarding bacterial *in vivo* gene expression is complementation. As mentioned previously, although SCOTS technique shows a tremendous advance in bacterial *in vivo* gene expression, its combination with cDNA microarrays gives more valuable genetic data. Therefore, a continuing progress made in improving the techniques to acquire genetic information regarding *in vivo* gene expression is still needed, and the advances will likely be gradual improvements in current technologies rather than new technologies.

### 2.9 REFERENCES


CHAPTER 3

MORAXELLA OSLOENSIS GENE EXPRESSION IN THE PRIMITIVE MOLLUSK HOST DEROCERAS RETICULATUM

3.1 ABSTRACT

**Background:** Slugs, particularly *Deroceras reticulatum*, are regarded as one of the important primitive invertebrates. The bacterium *Moraxella osloensis* is a mutualistic symbiont of the slug-parasitic nematode *Phasmarhabditis hermaphrodita*. Naturally, *P. hermaphrodita* vectors *M. osloensis* into the shell cavity of *D. reticulatum* which multiplies and kills slugs. As *M. osloensis* is the main killing agent, genes expressed by *M. osloensis* in slugs are likely to play important roles in virulence. Studies on pathogenic interactions between bacteria and primitive hosts are few, but such studies have the potential to shed light on the evolution of bacterial virulence. Therefore, we
investigated such an interaction by determining gene expression of *M. osloensis* in its primitive mollusk host *D. reticulatum* by selectively capturing transcribed sequences.

**Results:** Thirteen genes were identified to be up-regulated post infection. Compared to the *in vitro* gene expression in stationary phase, we found that ubiquinone synthetase (*ubiS*) and acyl-coA synthetase (*acs*) genes were up-regulated in both *D. reticulatum* and stationary phase *in vitro* cultures, but the remaining 11 genes were exclusively expressed in *D. reticulatum* and were infection specific. Mutational analysis on genes of protein-disulfide isomerase (*dsbC*) and *ubiS* suggested a causal role for both genes in the virulence of *M. osloensis* to the slug, and we further confirmed that UbiS is important to *M. osloensis* growth and DsbC serves as a virulence factor by measuring the *in vitro* growth rate of mutants.

**Conclusion:** Distribution of these genes in various bacterial pathogens indicates that although virulence genes are conserved, far fewer genes may be expressed in a primitive host, and further, *dsb* gene family may be one of the most ancient bacterial virulence genes.

### 3.2 BACKGROUND

The dialog between a host and bacterium requires the coordinated activity of many bacterial gene products in response to the host (Chiang *et al.*, 1999). As bacterial pathogens infect a wide variety of evolutionarily distinct hosts, including both lower and higher eukaryotes, investigating the bacterial pathogenesis in a diverse set of hosts can contribute to our understanding of the evolution of bacterial virulence. There have been
extensive studies on bacteria pathogenic to higher order hosts (Brodgen et al., 2000; Donnenberg, 2000; Hueck, 1998), particularly the fish, bird and mouse (Coady et al., 2006; Dozois et al., 2003; Fryer & Hedrick, 2003; Osorio et al., 2004; Romalde, 2002; Thomas-Jinu & Goodwin, 2004; Vazquez-Juarez et al., 2003; Yeh et al., 2005), consequently zebrafish and mouse have been selected as model animals for the study of bacterial pathogenesis (Cosma et al., 2006; Neely et al., 2002; Pradel & Ewbank, 2004). These studies have provided ample information on the virulence genes essential for pathogenesis, yet we know little about the origin and evolution of these genes. While there are several reports of bacterial illness in snakes, tortoises, and reptiles (Dickinson et al., 2001; Isaza et al., 2000; McLaughlin et al., ; Merchant et al., 2006; Tu et al., 2005), there has been no systematic study of bacterial pathogenesis in primitive hosts. Compared to the studies on bacterial pathogenesis in vertebrates and plants, little is known about bacteria-involved infections in primitive hosts with poorly developed immune systems such as mollusks (Paillard et al., 2004). Therefore, characterizing the bacterial genes expressed in a host with a primitive immune system will enable us to gain a better understanding of the evolution of bacterial virulence (Chiang et al., 1999).

As primitive invertebrates, mollusks have been proven to be excellent model systems for studies in neurophysiology, behavioral ecology and population genetics (Barker, 2001). Moraxella osloensis is a gram-negative, oxidase positive, aerobic bacterium within the family Moraxellaceae in the gamma subdivision of the purple bacteria. This bacterium has recently been identified as one of the natural symbionts of a bacteria-feeding nematode, Phasmarhabditis hermaphrodita (Rhabditida: Rhabditidae),
which is a lethal endoparasite of slugs (Wilson *et al*., 1995; Wilson & Grewal, 2005),
including the introduced gray garden slug *Deroceras reticulatum* (Wilson *et al*., 1993).
Naturally, bacteria colonize the gut of nematode infective juveniles (IJs) which represent
a specialized stage of development adapted for survival in the unfavorable environment.
The IJs seek out and enter the slug’s shell cavity through the posterior mantle region.
Once inside, the bacteria are released, and the IJs resume growth feeding on the
multiplying bacteria (Tan & Grewal, 2001a; Tan & Grewal, 2001b; Wilson *et al*., 1993).
The infected slugs die in 4-10 days, and the nematodes colonize the entire cadaver and
produce next generation IJs which leave the cadaver to seek a new host (Wilson *et al*.,
1993).

The lethality of these nematodes to slugs has been shown to correlate with the
number of *M. osloensis* cells carried by IJs (Tan & Grewal, 2001b). Tan and Grewal
(2001b) demonstrated that the 72 h old *M. osloensis* cultures inoculated into the shell
cavity were highly pathogenic to the slug. They further reported that *M. osloensis*
produced an endotoxin which was identified to be a rough type lipopolysaccharide (LPS)
with a molecular weight of 5300 KD, and the purified *M. osloensis* LPS was toxic to the
slug with an estimated 50% lethal dose of 48 μg when injected into the shell cavity (Tan
& Grewal, 2002).

Although these studies laid the foundation for the bacteria–slug interaction, the
virulence mechanisms of *M. osloensis* that result in pathogenesis and slug mortality are
not established. The present study was designed to examine the molecular and genetic
basis of *M. osloensis* virulence to the slug *D. reticulatum*. Several techniques have been
developed to study bacterial gene expression specifically in host during infection. The most commonly used techniques include in vivo expression technology (IVET) (Mahan et al., 1993), signature-tagged mutagenesis (STM) (Hensel et al., 1995), and differential fluorescence induction (DFI) (Valdivia & Falkow, 1997). Recently, the selective capture of transcribed sequences (SCOTS) technique has been developed to study bacterial gene expression in macrophage (Graham & Clark-Curtiss, 1999). This technique has been used to identify bacterial virulence factors, and has been demonstrated to be sensitive enough to isolate genes expressed in specific tissues of infected animals (Baltes & Gerlach, 2004; Dozois et al., 2003). As SCOTS technique can easily differentiate between host and bacterial cDNAs, and does not require prior genetic information of the pathogen, we applied this technique to determine M. osloensis gene expression in the mollusk host D. reticulatum at two time points following infection. Because important changes in gene expression can occur in bacteria during transition from active growth to stationary phase (Dozois et al., 2003; Tortora et al., 2004), we also investigated differential gene expression of 72 h M. osloensis in the in vitro cultures (stationary phase) relative to 24 h cultures (log phase) to confirm the infection specificity of the in vivo expressed genes. Mutational analyses (virulence of mutants to the slug and in vitro growth rate of mutants) were also carried out to examine the roles of several identified genes.
3.3 RESULTS

Post inoculation *M. osloensis* counts varied from $10^5$ to $10^8$ colony-forming units (CFU) per *D. reticulatum* slug. Transcripts expressed by *M. osloensis* within the slug at 48 h and 96 h post inoculation were identified by subjecting the *in vivo* cDNAs to three iterations of SCOTS in the presence of the transcripts expressed by 48 h *in vitro* cultured *M. osloensis*. The SCOTS enriched cDNAs represented the differentially expressed genes within the slug but in lower abundance or absence in 48 h *in vitro* cultures.

**Analysis of Differentially In vivo Expressed Genes.** The enriched cDNAs were further screened by dot blot hybridization (Fig. 1). A total of 97 clones (27 from 48 h post inoculation, and 70 from 96 h post inoculation) that hybridized exclusively to the cDNA probes prepared from *in vivo* cDNAs were chosen for nucleotide sequencing and analysis. A fraction of the identified sequences was further confirmed to be *M. osloensis* specific by PCR amplification of *M. osloensis* genomic DNA with the designed primers based on identified sequences, and the results confirmed that *M. osloensis* transcripts were successfully isolated from the infected slugs using the SCOTS technique. The identified sequences were analyzed using the non redundant algorithms of BLAST in NCBI (National Center for Biotechnology Information). In this study, the identity of up-regulated sequences at 48 h and 96 h post infection was identical. We identified 13 distinct up-regulated sequences (Table 3.1) which were identified to carry putative functions in cell structure integrity, energy metabolism, degradation, and translocation. For each identified sequence, about two to five cDNA molecules were detected in the
screened library, and these clones showed the same hits in the GenBank. Thus, it may predict that libraries of up-regulated genes are redundant and most *M. osloensis* up-regulated genes within the slug have been identified. Among the identified sequences, three of them encoded for hypothetical proteins with unknown functions, and further analysis by inverse PCR amplification and sequencing demonstrated that they were similar to the sequences with functions of preprotein translocase (SecA) (*M11*), acyl-coA synthetases (Acs) (*M12*), and acetyl-coA carboxylase (Acc) (*M13*). One of the sequences (*M3*) did not exhibit similarity to any genes or gene products in current databases, and is possibly novel. The *in vivo* captured sequences arbitrarily termed, *M1*, *M2*, and *M4*, corresponded to membrane proteins, and one of the features for these sequences was the presence of “GDPG” repeat. Sequence *M5* shows similarity with the protein-disulfide isomerase (DsbC) in other bacteria. Sequences *M6*, *M8*, and *M10* have similarity to proteins that encode iron regulation related genes in other bacteria; thus, the identification

<table>
<thead>
<tr>
<th>Seqs</th>
<th>Genes</th>
<th>Accession</th>
<th>Identity, Organism</th>
<th>Possible function</th>
</tr>
</thead>
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<tr>
<td><em>M1</em></td>
<td><em>sclB</em></td>
<td>DQ324274</td>
<td>50%, <em>Streptococcus</em> sp.</td>
<td>Variable surface lipoprotein</td>
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<tr>
<td><em>M2</em></td>
<td><em>vspC</em></td>
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<td>46%, <em>Mycobacterium</em> sp.</td>
<td>Alanine and proline rich protein</td>
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<td>Preprotein translocase</td>
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<tr>
<td><em>M12</em></td>
<td><em>acs</em></td>
<td>DQ324263</td>
<td>76%, <em>Psychrobacter</em> sp</td>
<td>Acyl-CoA synthetases</td>
</tr>
<tr>
<td><em>M13</em></td>
<td><em>acc</em></td>
<td>DQ324264</td>
<td>86%, <em>Psychrobacter</em> sp</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td><em>M4</em></td>
<td><em>spp</em></td>
<td>DQ324275</td>
<td>54%, <em>Lactobacillus</em> sp.</td>
<td>Surface protein precursor</td>
</tr>
<tr>
<td><em>M5</em></td>
<td><em>dsbC</em></td>
<td>DQ324260</td>
<td>49%, <em>Psychrobacter</em> sp</td>
<td>Protein-disulfide isomerase</td>
</tr>
<tr>
<td><em>M6</em></td>
<td><em>abp</em></td>
<td>DQ324271</td>
<td>79%, <em>Acinetobacter</em> sp</td>
<td>ATP-binding protein</td>
</tr>
<tr>
<td><em>M7</em></td>
<td><em>ats</em></td>
<td>DQ324266</td>
<td>31%, <em>Psychrobacter</em> sp</td>
<td>Acetyltransferases</td>
</tr>
<tr>
<td><em>M8</em></td>
<td><em>ubiS</em></td>
<td>DQ324261</td>
<td>74%, <em>Psychrobacter</em> sp</td>
<td>Ubiquinone synthesis</td>
</tr>
<tr>
<td><em>M9</em></td>
<td><em>pca</em></td>
<td>DQ324272</td>
<td>67%, <em>Psychrobacter</em> sp</td>
<td>Carboxymuconolactone decarboxylase</td>
</tr>
<tr>
<td><em>M10</em></td>
<td><em>adh</em></td>
<td>DQ324273</td>
<td>90%, <em>Acinetobacter</em> sp</td>
<td>Aldehyde dehydrogenase</td>
</tr>
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<td><em>M3</em></td>
<td></td>
<td></td>
<td>No similarity</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Table 3.1:** SCOTS identified *M. osloensis* genes differentially expressed *in vivo*
of these genes suggests that iron availability in slug host may be limited. The sequence of \textit{M7} shares similarity to acetyltransferase (Ats) that functions in the energy metabolism and is often involved in antigen synthesis. The sequence \textit{M9} has similarity with the predicted \(\beta\)-carboxymuconolactone decarboxylase (PCA) which encodes enzymes participating in the conversion of protocatechuate to succinate and acetylcoenzyme A (Acyl-CoA).

\textbf{Analysis of differentially \textit{in vitro} expressed genes at the stationary phase.} In order to determine if the identified \textit{in vivo} expressed genes are infection-specific, the differential expression of transcripts corresponding to 72 h \textit{M. osloensis} cultures (stationary phase, data not shown) relative to 24 h cultures (early-log phase) was examined by SCOTS technique. Nine fragments (descriptive \textit{Mo1}, \textit{Mo2}…\textit{Mo9}) were identified to be differentially expressed in 72 h relative to 24 h cultures (Table 3.2). Eight of these were similar to protein sequences from other bacteria, while one locus (\textit{Mo9}) did not show any similarity in NCBI databases, and was thus possibly novel for \textit{M. osloensis}. Fragments \textit{Mo2} and \textit{Mo4} have similarity to transposases and integrase, \textit{Mo6} is similar to topoisomerase, \textit{Mo1} has high similarity to Actin-like ATPase, and \textit{Mo7} is similar to ATPase involved in DNA replication. Two genes, ubiquinone synthetase (UbiS) and acyl-coA synthetase (Acs), that were found to be differentially expressed under \textit{in vivo} condition (see above), were also found to be differentially expressed in the 72 h \textit{in vitro} relative to the 24 h \textit{in vitro} cultures. Thus, these two genes are not infection specific.
<table>
<thead>
<tr>
<th>Seqs</th>
<th>Gene</th>
<th>Accession</th>
<th>Identity, Organism</th>
<th>Possible function</th>
</tr>
</thead>
<tbody>
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<td>DQ904630</td>
<td>87%, <em>Flavobacterium</em> sp.</td>
<td>Actin-like ATPase</td>
</tr>
<tr>
<td>Mo2</td>
<td>tra</td>
<td>DQ904631</td>
<td>72%, <em>Psychrobacter</em> sp.</td>
<td>Transposase</td>
</tr>
<tr>
<td>Mo3</td>
<td>ubiS</td>
<td>DQ904632</td>
<td>97%, <em>Moraxella</em> sp.</td>
<td>Ubiquinone synthesis</td>
</tr>
<tr>
<td>Mo4</td>
<td>int</td>
<td>DQ904633</td>
<td>78%, <em>Psychrobacter</em> sp.</td>
<td>Integrase</td>
</tr>
<tr>
<td>Mo5</td>
<td>top</td>
<td>DQ324278</td>
<td>80%, <em>Psychrobacter</em> sp.</td>
<td>Conserved protein</td>
</tr>
<tr>
<td>Mo6</td>
<td>pol</td>
<td>DQ324269</td>
<td>71%, <em>Psychrobacter</em> sp.</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td>Mo7</td>
<td>pol</td>
<td>DQ904634</td>
<td>38%, <em>Psychrobacter</em> sp.</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>Mo8</td>
<td>acs</td>
<td>DQ904635</td>
<td>64%, <em>Acinetobacter</em> sp.</td>
<td>Acyl-CoA synthetase</td>
</tr>
<tr>
<td>Mo9</td>
<td></td>
<td>DQ904636</td>
<td>No similarity</td>
<td>unknown</td>
</tr>
</tbody>
</table>

**Table 3.2:** SCOTS identified genes exclusively expressed by 72h *in vitro* cultures

**Mutational Analysis of Identified Genes.** The potential role in virulence of selected genes transcribed by *M. osloensis* in the slug was further investigated. Among the 13 identified genes, we inactivated 3 genes and tested the virulence of the resulting mutants to the slug. These genes were selected for functional analysis because they represent three distinct groups: cell surface protein (*M4*) which has been previously reported as a virulence gene in most pathogenic bacteria, protein-disulfide isomerase (DsbC) (*M5*) which has been recently identified as a virulence gene in pathogenic bacteria associated with higher order hosts, and ubiquinone synthetase (UbiS) (*M8*) which has been reported to be important to bacterial survival but not virulence. Isogenic mutations were constructed in *M. osloensis* by insertion-deletion strategy using inverse PCR with the aid of engineered *Ape* I and *Bma* I sites, and the resulting isogenic mutants were obtained *in vitro* after 72-h incubation, and they were termed as *M-M4*, *M-M5*, and *M-M8*, respectively. The virulence of wild type and mutants was determined by direct inoculation into the slug *D. reticulatum* and subsequent survival analysis. Slug mortality
caused by mutants mainly occurred in the first two days, and all three mutants demonstrated the level of virulence similar to the wild-type strain in the first two days (Fig. 2). However, after 4 days of infection, all three mutants produced significantly lower slug mortality relative to the wild-type parent strain.

We further complemented the constructed mutants. The full length and promoter region of $M5$ and $M8$ were obtained by inverse PCR strategy with the aid of six pairs of primers (data not shown) and six restriction enzymes ($Ape$ I, $EcoR$ I, $Hinf$ I, $Pst$ I, $Bma$ I, and $Bxa$ I). Amplification of the full length open reading frame (ORF) of $M4$ was not successful due to the presence of repetitive sequences, and thus was not used for further analysis. The ORF of $M5$ and $M8$ were 843bp and 1671bp, and the blast searches confirmed that they were similar to protein-disulfide isomerase and ubiquinone synthesis genes, respectively. The sequence analysis revealed that $M5$ gene had an N-terminal signal peptide, and contained a thioredoxin-like domain with the characteristic Cys-x-x-Cys active site. The complemented mutants, termed as $C-M5$ and $C-M8$, were obtained by transferring intact $M5$ and $M8$ genes borne in the plasmid into the respective $M. osloensis$ mutant by electroporation. The level of virulence of complemented mutants in the slug was similar to wild-type strains.

**Growth rates of mutants.** As reduced virulence of mutants may result from growth defect, the growth rates of DsbC ($M5$) and UbiS ($M8$) mutants in the BHI medium were examined (Fig. 3). Compared to the wild-type $M. osloensis$, the DsbC mutant did not show any growth defect *in vitro*. However, the UbiS mutant showed significant
reduction in growth rate \textit{in vitro}. The UbiS mutant grew slower than the wild type and its viability (shown as OD600) was about 12\% lower relative to the wild-type strain after 12 h (Fig. 3), and this defect was fully complemented. In addition, the growth defect phenotype became evident at the time of the late log phase preparing to enter into the stationary phase.

\section*{3.4 DISCUSSION}

The current study is the first description of virulence genes expressed by a bacterium in a primitive host - the mollusk. The SCOTS analysis demonstrates that \textit{M. osloensis} expressed 13 genes exclusively in the slug host when compared with bacteria growing \textit{in vitro} in the log phase. Comparison with differentially expressed genes by 72 h relative to 24 h \textit{in vitro} cultures revealed that 11 of the 13 genes were infection specific.

Among the SCOTS identified \textit{in vivo} expressed genes, three genes Abp, UbiS, and PCA (\textit{M6, M8,} and \textit{M9}) are similar to the proteins important for bacterial survival, and of which, Abp is similar to ATP binding proteins that confer multiple resistance and pH adaptation. Blast search reveals that Abp also shares high similarity to the polysaccharide export protein. In the case of pathogenic bacteria, polysaccharide is often secreted as an evolutionary adaptation to adhere to host surfaces and to escape from drying and host immune defense (Boulnois & Jann, 1989; Moxon & Kroll, 1990). Therefore, it is possible that Abp may play an important role in \textit{M. osloensis} survival within the slug host. Also the identification of PCA sequence that has similarity to \(\beta\)-carboxymuconolactone decarboxylase gene suggests a role for PCA in \textit{M. osloensis}
survival within the slug as it can serve as both nutritional and detoxification functions in other bacteria (Orville et al., 1997). UbiS is similar to the protein sequence with function of ubiquinone synthesis. Ubiquinone is a central component of the electron transport chain under aerobic conditions and functions in the formation of disulfide bonds in periplasmic proteins by facilitating the reoxidation of protein-disulfide isomerase (Alexander & Young, 1978; Sheehan et al., 2003). While there is no report showing the role of uniquinone synthetase (UbiS) gene in virulence of bacterial pathogens from higher order hosts, reduced slug mortality following inactivation of UbiS gene confirmed its importance in *M. osloensis* virulence to the slug. In addition, UbiS gene was also up-regulated in the stationary phase, which indicated that this gene was important to *M. osloensis* survival in the suppressive environment and could be induced by the stress condition. Further, examination of UbiS mutant growth *in vitro* indicated that the reduced virulence of this mutant may be due to the reduced growth rate.

Preproteins are exported primarily across the cytoplasmic membrane to the periplasm or the outer membrane by the sec system composed of the translocase SecYEG (a protein-conducting channel) and SecA (an ATP-dependent motor protein) (Economou, 1998; Vrontou & Economou, 2004; Wickner & Schekman, 2005). One of the identified genes *M11* is similar to the bacterial preprotein translocase SecA. The SecA interacts dynamically with the SecYEG components to drive the transmembrane movement of newly synthesized preproteins (Mori & Ito, 2001). Tomkiewicz et al. (Tomkiewicz et al., 2006) showed that in *Escherichia coli* SecA drives a constant rate of preprotein translocation consistent with a stepping mechanism of translocation. Further, the SecA
expression is subject to the translational control in response to the cellular activity of protein translocation (Mori & Ito, 2001; Nakatogawa & Ito, 2001; Sarker et al., 2000). In this study, up-regulation of this gene in vivo may indicate that the expression of SecA can be regulated by the infection process, and thus *M. osloensis* is able to export secretory proteins constantly in the slug host.

Three of the identified sequences *M1*, *M2*, and *M4* are similar to the structural proteins of the outer membrane. Outer membrane proteins are known to be critical for establishment of disease in the host by causing resistance to immune killing (Rasmussen & Bjorck, 2001; Whatmore, 2001) as demonstrated in a fish pathogenic bacterium, *Edwardsiella tarda* (Rao et al., 2003). Virulence of *M4* mutant to the slug is significantly reduced indicating that surface membrane proteins are also important for *M. osloensis* infection in the slug.

Genes of Ats (*M7*), Adh (*M10*), Acs (*M12*), and Acc (*M13*) are similar to proteins that are reported to be important virulence related genes in other bacterial pathogens. Gene Ats is similar to the sequence of acetyltransferase which has been known as an effector protein (YopJ) in bacterium *Yersinia* (Mukherjee et al., 2006), and plays an important role in regulating biological signaling. Gene Ald is similar to the sequence of aldehyde dehydrogenase (AldH). In *Vibrio cholerae*, AldH gene has been found to be located in a pathogenicity island in epidemic and pandemic strains but absent from non-pathogenic strains (Karaolis et al., 1998). SCOTS identified Acs shares similarity with long chain fatty acid CoA synthetase (FadD), an enzyme involved in lipid synthesis and whose expression may be important at various stages of infection (Black et al., 2000;
Gargiulo et al., 1999; Greenway & Silbert, 1983; Hill et al., 2003). FadD gene expression has been demonstrated to be important in virulence in a number of organisms, including Xanthomonas campestris (Soto et al., 2002) and Salmonella enterica serovar Typhimurium (Utley et al., 1998). In this study, Acs gene was also identified to be up-regulated in vitro at the stationary phase. Thus, the expression of Acs protein may be related to M. osloensis survival and virulence under in vivo conditions.

The gene DsbC (M5), protein-disulfide isomerase, belongs to the gene family Dsb and is involved in disulfide bond exchange which catalyses the folding of various factors including virulence determinants such as the components of type III secretory machinery and assembly of type II secreted subunits into effector proteins in a number of bacteria (Sandkvist, 2001; Yu, 1998; Yu & Kroll, 1999). Various gram-negative bacterial pathogens use type II or III secretion system as a basic virulence mechanism (Hueck, 1998). Dsb protein has been identified as a virulence factor in diverse bacterial pathogens associated with different higher order hosts. In pathogenic Shigella flexneri, Dsb gene is necessary for intracellular survival and cell-to-cell spread in the host (Yu & Kroll, 1999). In the fish pathogenic bacterium, Flavobacterium psychrophilum, mutants of Dsb-like protein gene exhibited reduced virulence and cytotoxicity (Alvarez et al., 2006). Dsb gene was also identified as a virulence factor by SCOTS technique in the pig pathogenic bacterium Actinobacillus pleuropneumoniae (Baltes & Gerlach, 2004). Mutation of DsbC in Bordetella pertussis resulted in decreased toxin secretion (Stenson & Weiss, 2002). In our study, the attenuated virulence plus the normal growth rate of M5 mutant proves that DsbC serves as a virulence factor in M. osloensis in the slug host.
Previous studies by Tan and Grewal (2002) showed that lipopolysaccharide (LPS) of *M. osloensis* alone was sufficient to cause slug mortality. Compared to the virulence defects of DsbC and UbiS mutant, it may hypothesize that there is relationship between LPS and DsbC or UbiS product despite the current lack of experimental evidence. The link between UbiS and Dsb has been reported from the study in *Escherichia coli* that disulfide bond formation in exported proteins is catalyzed by Dsb, and is directly coupled to the electron transport chain via reoxidation of Dsb by either ubiquinone or menaquinone (Kadokura *et al.*, 2000). Based on the fact that LPS is recognized by host factors, such as Toll-like receptors which are often characterized by the disulfide bonds, to induce host immune response (Diks *et al.*, 2001; Freudenberg *et al.*, 2001; Palsson-McDermott & O’Neill, 2004; Raetz & Whitfield, 2002; Zweigner *et al.*, 2006), we suggest that DsbC product can help LPS avoid host recognition by destroying the host disulfide bonds. In addition, most animals possess agglutinating activity to recognize self or non-self particles, and the agglutinins are believed to be lectins in invertebrates. According to Barker (2001), the land slug *Incilaria* contains three lectins which show significant sequence similarity to other animals, having two disulphide bonds which may be related to lectin activity. Since the Dsb gene family in prokaryotes has the ability to rearrange non-native disulfides to their native configuration (Nakamoto & Bardwell, 2004), it is proposed that DsbC functioning as a virulence factor in *M. osloensis* may be related to the host self/non-self recognition.
3.5 CONCLUSION

In this study, no differences in *M. osloensis* gene expression between 48 h and 96 h post inoculation were observed indicating that the infection was established in 48 h. Unlike the bacterial pathogens of vertebrates (Baltes & Gerlach, 2004; Dozois *et al.*, 2003; Graham *et al.*, 2002) in which over 25 bacterial genes have been shown to be differentially expressed in each host using the same SCOTS technique, the number of *M. osloensis* genes up-regulated in the slug is small. Thus, we suggest that in parallel to the evolution of the host defense system, the spectrum of gene expression in the bacterial pathogens may have increased. Our study also suggests that some virulence determinants such as Dsb, may have ancient roots.

3.6 METHODS

**Bacteria, Slugs, and Culture Conditions.** A type strain of *Moraxella osloensis* acquired from the American Type Culture Collection (ATCC) was used in this study. The bacteria were cultured in Brain Heart Infusion (BHI) (Difco) broth at 25°C according to the manufacturer’s instructions, and confirmed to be *M. osloensis* by 16S rDNA sequencing. The association between this bacterial strain and the nematode *P. hermaphrodita* had been previously evaluated by assessing the recovery of infective juveniles in the bacterial cultures. The growth rate of *M. osloensis* was determined by turbidity measurements according to Tortora et al. (Tortora *et al.*). Slug *D. reticulatum* adults were collected from the field and fed on pieces of fresh carrots at 18°C for at least 12 days. All the slugs were fed on sterile water for three days to wash the intestine
before bacterial infection. Only healthy actively moving adult slugs were used in experiments.

**General Techniques.** Bacterial genomic DNA was prepared using standard method for Gram negative bacteria (Sambrook *et al.*). Biotinylation of bacterial genomic DNA was obtained with EZ-Link Psoralen-PEO-Biotin (Pierce) according to the manufacturer’s instructions. The total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were treated with RNase-free DNase I (Ambion, Austin, TX) according to the manufacturer’s guidelines, and were concentrated by spectrophotometer and gel electrophoresis. Total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase (Invitrogen RT-PCR kit) according to the manufacturer’s instructions. First strand cDNA was made double-stranded with Klenow fragment (NEB, Beverly, MA) as described by Froussard (Froussard, 1992). Restriction endonucleases and ligase enzymes (Promega) were used according to the manufacturer’s guidelines. The DNA samples were sequenced at the Biotechnology Center, Madison, WI, USA; and McGill University and Genome Quebec Innovation Centre, Montreal, Canada. Sequence analysis was carried out using BLAST algorithms (blastx and tblastx) in GenBank NCBI.

**SCOTS Analysis.** We extended SCOTS technique to determine the *in vivo* gene expression of *M. osloensis* within the slug post infection at two different time points. Briefly, for the control, 5μg total RNA samples obtained from 48 h *M. osloensis* cultures
(OD600=0.8) were converted to cDNAs and then made double-stranded. Primers (SCOT09) with a defined sequence (SCOT0) at the 5’ end and random sequence at the 3’ end were used for both first and second strand cDNA synthesis (Table 3.3). cDNAs were then PCR amplified by using the defined primers (SCOT0) for 30 cycles. Bacterial cDNA normalization was done as described by Graham and Clark-Curtiss with some modifications (Graham & Clark-Curtiss, 1999). In this study, the ribosomal operon (rDNA) of *M. osloensis* was amplified using primers Mor-F and Mor-R (Table 3.3), and a plasmid containing the amplified operon was constructed using TOPO XL PCR Cloning kit (Invitrogen) and was used to block the abundant rRNA sequence in order to effectively capture the cDNA molecules representing mRNA transcripts. The rDNA operon was added to biotinylated genomic DNA at a ratio of 10:1. The genomic DNA-rDNA mixture was sonicated to a size range of 1 to 5kb. The sonicated, biotinylated genomic DNA-rDNA mixture containing 6μg rDNA and 0.6μg genomic DNA was denatured and hybridized for 30 min at 67°C. PCR amplified cDNA (6μg) from 48 h cultures was denatured and added to the genomic DNA-rDNA prehybridized mixture,

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<tr>
<td>SCOT1109</td>
<td>5'-ATGCCGAAATCCAGTCAAGANNNNNNNNNNN-3</td>
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**Table 3.3:** Primers used in SCOTS analysis
and hybridized at 67°C for 24 h. Streptavidin magneSphere paramagnetic particles (Promega) were used to capture the bacterial cDNA that hybridized to biotinylated genomic DNA according to the manufacturer’s instructions. Captured cDNA was then eluted, precipitated, and amplified by PCR with the defined primers for additional two successive rounds of SCOTS. The finally amplified cDNA mixtures were considered to be normalized in vitro cDNAs. For the treatment, 50μl suspension of 48 h *M. osloensis* cultures (OD 600 = 0.8) containing 5 x 10^7 cells according to the bioassay test (Tan & Grewal, 2001b) was inoculated into the shell cavity of slug *D. reticulatum*. Inoculation into the shell cavity is mimicking the natural infection of the slug by nematode-bacteria complex. Before RNA isolation, a parallel experiment was performed to evaluate the number of viable *M. osloensis* in the slug post inoculation. In brief, the infected slug was surface sterilized by immersing in 0.1% thimerosal for 3 h, washed by sterile water, and mortared. The slug homogenate was serially (10-fold) diluted and plated on Mueller-Hinton II agar (MHAII; BBL Becton Dickinson, Cockeysville, Md.) (Vaneechoutte et al., 2000). Numbers of colony forming units (CFU) were then counted based on the oxidase reaction. After that, the total RNA was isolated from the survived slug at 48 h and 96 h post-infection. Three 5μg total RNA samples obtained from three infected slugs were pooled and made double-stranded by the random primer (Primer SCOT189 was used for post 48 h infection, and SCOT1109 was used for post 96 h infection). Double-stranded cDNAs were then PCR amplified for 30 cycles by the defined primer (Primer SCOT18 was used for post 48 h infection, and SCOT110 was used for post 96 h infection). Amplified cDNAs from 48 h or 94 h post infection were added to the genomic DNA-
rRNA prehybridized mixture to hybridize at 67°C for 24 h. The bacterial cDNAs that hybridized to bacterial genomic DNA were captured by streptavidin-coated magnetic particles. The captured cDNAs were eluted and PCR amplified. In the first round of SCOTS, three separate samples of cDNA were captured by hybridization to biotinylated genomic DNA in parallel reactions. After the first round of SCOTS, the three amplified cDNA preparations for 48 h or 96 h post infection were combined, denatured, and again hybridized to genomic DNA-rDNA mixture for two successive rounds of SCOTS. The finally amplified cDNA mixtures were considered to be normalized in vivo cDNA molecules. To enrich the normalized in vivo cDNAs, the normalized cDNAs from 48 h or 96 h post infection were hybridized to biotinylated genomic DNA that has been prehybridized with both rDNA and normalized in vitro cDNAs. After hybridization, the bacterial cDNAs were captured and PCR amplified for next round of enrichment. Finally, the enriched bacterial cDNAs were cloned into the original TA cloning kit (Invitrogen). Individual cloned inserts were screened by dot blot hybridization with probes made from cDNA mixtures obtained by SCOTS either from in vivo or in vitro. Clones that only hybridized to the probe made from normalized in vivo cDNAs were chosen for sequence analysis. The selected inserts were sequenced and analyzed.

Furthermore, in order to determine that the identified in vivo expressed genes were specific to infection or suppressive condition, we examined the differential in vitro gene expression of 72 h M. osloensis cultures (stationary phase) relative to 24 h cultures (early-log phase) using the similar procedure.
Inactivation of SCOTS Identified *In vivo* Expressed Genes. Several *M. osloensis* mutants of SCOTS identified genes were constructed with insertion-deletion strategy by inverse PCR according to Furano and Campagnar (2003). Briefly, the SCOTS identified cDNA sequence was cloned into a PGEM-T vector (Promega). The primers with engineered restriction sites were used in the inverse PCR to amplify the cDNA fragment. This resulted in a deletion of about 50bp nucleotides internal to SCOTS identified sequence. The nonpolar kanamycin resistance gene was amplified from a TA vector with the engineered primers. Inverse PCR product and kanamycin resistance gene were subjected to restriction digestion and ligation, resulting in the plasmid containing the selected sequence and kanamycin resistance gene. Sequence analysis was performed to confirm the proper insertion of the cassette. The resulting plasmid was PCR amplified, and product was used to naturally transform wild-type *M. osloensis* (Furano & Campagnar, 2003; Juni, 1974). Insertion of the kanamycin gene through a single recombination event resulted in the disruption of the wild-type gene. In brief, a 100μl aliquot of an early-log phase *M. osloensis* culture was plated onto BHI agar, and 20ng of the purified mutagenesis constructed sequence from above was spotted onto a portion of the bacterial lawn. After incubation for 6 h under standard growth conditions, the area of the bacterial lawn that had been inoculated with the constructed sequence was swabbed onto BHI agar plates containing kanamycin or added to BHI broth (broth method) containing kanamycin and slug homogenate. Samples from broth method were incubated for 12 h, and then different dilutions were spot inoculated on the selective plates containing kanamycin to select the mutants. Chromosomal DNA from mutants was
isolated and subjected to PCR analysis as well as sequence analysis to confirm the integration of the inactivated genes into the genome. The stability of these mutants was verified by growing the mutated strains in medium lacking kanamycin and the frequency of kanamycin resistance, compared with total cell count, was then determined.

Due to the limited genetic information for *M. osloensis*, we used inverse PCR strategy to amplify the full sequence of SCOTS captured sequences. The amplified sequences were further analyzed by the bio-software such as SignalP 3.0 (Bendtsen et al., 2004). The cloned intact genes were cloned into pCR2.1 using the TA Cloning kit (Invitrogen) according to the manufacturer’s instructions. The plasmid containing the intact genes was then transferred into the responded mutant and wild-type backgrounds by electroporation. The complemented mutants were further confirmed before virulence test. The virulence of wild type, mutants, and complemented mutants of *M. osloensis* cultures were determined by quantifying slug mortality following the injection of 72 h bacterial culture into the shell cavity as described below.

**Virulence of *M. osloensis* to the Slug.** The virulence of *M. osloensis* cultures was determined by quantifying slug mortality following the injection of bacteria into the shell cavity. The different bacterial cells were precipitated and washed several times using a sterile saline solution (0.85% NaCl), and the total numbers of bacteria in each suspension were measured with a spectrophotometer at a wavelength 600 nm. Different *M. osloensis* cultures were injected into the slug shell cavity with the same dose used in the SCOTS analysis, and which is consistent with the dose used by Tan and Grewal (2001b). Slugs
injected with the saline solution served as controls. Six slugs were tested in each
treatment, and three replicas were used for each treatment. Slug mortality was recorded 4
days after inoculation at 18°C. The survival analysis was plotted as Kaplan-Meier plots
by using the statistical software GraphPad Prism 4 (GraphPad software Inc.), and the
Chi-square value for significance at each time point was calculated with significant
difference tests at P= 0.05.

Growth rate of mutants in the media. M. osloensis cells of wild type, mutants, and
complemented mutants were streaked on BHI agar plates and incubated at 25°C for 72 h.
Single colonies from the cultures were picked and inoculated into 3ml BHI broth, and
cultured at 25°C for 72 h. To obtain growth rate, 100ml BHI broth was inoculated with 1
ml of 72 h cultured bacterial cells, and the OD 600 value was recorded until the wild-type
bacteria reached the stationary phase.

List of abbreviations. IJs, infective juveniles; SCOTS, selective capture of transcribed
sequences

Author’s contributions. RA carried out performing the experiments, analyzing the data,
and writing the draft, and participated in the design of the study. SS carried out the
design of the study and participated in the coordination. PSG carried out the design of
the study and funding the research. All authors read and approved the final manuscript.
3.9 REFERENCES


Figure 3.1: A representative screening of enriched cDNAs by dot blot (Exposing time was 2 min). The enriched cDNAs were cloned into a TA vector. Each individual clone was screened with probes made either from SCOTS captured in vitro or in vivo cDNAs. The individual clones that only hybridize to the probe prepared from in vivo cDNAs (the dot with high density above the line) were chosen for further sequence analysis.
Figure 3.2: The slug survival post *M. osloensis* infection (plotted with Kaplan-Meier plots). The slug mortality caused by bacterial cultures is significantly different between wild type and mutants post 2-days infection. Contr, Saline; Wild, wild-type *M. osloensis*; M-Spp, mutants of Spp gene; M-DsbC, mutants of dsbC gene; V-M5, dsbC mutant containing the empty vector; CW-DsbC, wild-type *M. osloensis* containing the plasmid borne DsbC gene; C-M5, the complemented DsbC mutant; M-UbiS, mutants of UbiS gene; V-UbiS, UbiS mutant containing the empty vector; CW-UbiS, wild-type *M. osloensis* containing the plasmid borne UbiS gene; C-UbiS, the complemented UbiS mutant.
Figure 3.3: Growth curve for *M. osloensis* in culture derived from measurements of OD 600 over time. Wild: wild-type *M. osloensis*; M-DsbC: mutant of DsbC gene; C-DsbC: complemented DsbC mutant; M-UbiS: mutant of UbiS gene; C-UbiS: complemented UbiS mutant. All data are shown as the mean of three repeated experiments with standard errors.
CHAPTER 4

THREE NEW SUBSPCIES OF THE SYMBIOTIC BACTERIUM PHOTORHABDUS TEMPERATA: P. TEMPERATA SUBSP. BOEMAREI SUBSP. NOV., P. TEMPERATA SUBSP. FISCHERLESAUXII, AND P. TEMPERATA SUBSP. STACKEBRANDTII SUBSP. NOV.

4.1 ABSTRACT

Photorhabdus is a heterogeneous bacterial genus in Enterobacteriaceae. In this study, the bacterial symbiont from the entomopathogenic nematode Heterorhabditis bacteriophora GPS11 strain which has high virulence against several white grub species was isolated and characterized by 16S rRNA gene sequence and physiological properties. Partial 16S rRNA gene sequence showed 93% - 96% similarity to the published sequences from Photorhabdus temperata isolates which have been most commonly
associated with *Heterorhabditis megidis*. Unlike other strains of *P. temperata*, the GPS11 isolate produced acid only from glucose and inositol, utilized citrate as the carbon source, and displayed negative aesculin hydrolysis. Thus, this isolate represented a novel subspecies, hence the name *Photorhabdus temperata* subsp. *boemarei* subsp. nov. (type strain, ATCC 29304^T^), is proposed. Based on the comparative study and published genetic, phenotypic and geographic data on other *P. temperata* isolates, two additional subspecies are proposed. These subspecies are *P. temperata* subsp. *stackebrandtii* subsp. nov., (type strain proposed to be Meg isolate), which is associated with *H. megidis* isolated from North America and *P. temperata* subsp. *fischerlesauxii* (type strain, CIP 105563^T^), which is associated with *H. megidis* and *H. downesi* isolated from Europe, and besides the geographic differences these two subspecies are clearly separated by 16S rRNA sequences.

**Key Words:** *Photorhabdus temperata, Heterorhabditis bacteriophora*

4.2 INTRODUCTION

The bacteria *Photorhabdus* are symbiotically associated with the entomopathogenic nematodes (EPNs) *Heterorhabditis* (Boemare, 2002). Naturally, *Photorhabdus* bacteria colonize the gut of the nematode infective juveniles (IJs) which represent a specialized stage of development suited for survival in the unfavorable environment. The IJs actively seek out and penetrate insect hosts. Upon entry into the haemolymph, the IJs release the bacteria which multiply and secrete a wide range of
extracellular hydrolytic enzymes that serve to convert the organs and tissues of the insect into an ideal niche for nematode growth and development (Duchaud et al., 2003; Joyce et al., 2006). The insect dies of septicaemia, and the IJs recover into feeding fourth stage juveniles. These juveniles feed on symbiotic bacteria completing 1-3 generations in the host cadaver, and as food resources are depleted new IJs are produced which re-associate with the symbiotic bacteria and disperse in search of new hosts (Poinar, 1990).

The association between the bacterium *Photorhabdus* and the nematode *Heterorhabditis* is species specific. Usually *P. luminescens* is associated with *H. bacteriophora*, and *P. temperata* with *H. megidis*. *P. luminescens* is divided into three subspecies: *P. luminescens luminescens* isolated from *H. bacteriophora* Brecon group, *P. luminescens laumondii* isolated from *H. bacteriophora* HP88, and *P. luminescens akhurstii* isolated from *H. indica* from tropical and sub-tropical regions. Based upon 16S rRNA gene sequence information and metabolic properties, two new subspecies of *P. luminescens*, isolated from *H. bacteriophora*, *P. luminescens kayaii* and *P. luminescens thracensis* have been proposed (Hazir et al., 2004). There is some confusion for *P. temperata* because this species has been found associated with *H. megidis*, *H. zealandica*, and *H. downesi*. In addition, Hazir (2003) and Burnell and Stock (2000) reported that *P. temperata* was also harbored by *H. bacteriophora*. According to Boemare (Boemare, 2002), these reports do not modify the concept of one-to-one association between ENPs and their symbiotic bacteria species but represent problems of bacterial classification. In fact, *Photorhabdus* is a heterogeneous genus based on the DNA-DNA hybridization and
16S rRNA sequencing (Nielsen & Lubeck, 2002), and defining subspecies is necessary (Fischer-Le Saux et al., 1999).

In this study, we isolated and characterized a new subspecies of *P. temperata* associated with *H. bacteriophora* GPS11 stain using 16S rRNA sequence data and physiological traits. The GPS11 strain and its symbiotic bacteria possesses high virulence against several white grub species including *Popillia japonica*, *Anomala orientalis*, and *Cyclocephala borealis* (Grewal et al., 2002). As bacterial species can be separated by their physiological characters (Somvanshi et al., 2006), we compared our data with the published genetic and physiological data from other *P. temperata* isolates to establish classification of *P. temperata* associated with *Heterorhabditis* species.

### 4.3 MATERIALS AND METHODS

**Isolation of symbiotic bacteria.** The bacteria were isolated from the surface sterilized IJs of *H. bacteriophora* GPS11. The surface-sterilized IJs were suspended in 200 µl Brain Heart Infusion (BHI) broth and homogenized. Then the homogenate was streaked on the BHI agar plate for incubation at 28°C. After 48 h, all the bacterial colonies were analyzed for light production in the dark room and catalase activity by 3% hydrogen peroxide treatment. Only the bacterial colonies producing light and showing positive catalase activity were selected for further studies. The morphology of selected bacterial colonies was further observed by transferring to nutrient agar, Luria-Bertani (LB) agar, MacConkey agar, and NBTA indicator plate (per 1L distilled water: peptone 5.0g, beef
extract 3.0g, agar 15g, bromothymol blue 0.025g, and 2,3,5-triphenyl tetrazolium chloride 0.04g).

**Genotypic characterization.** The bacterial genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions. The primers 16SF (5'-CGAGCGGCGGACGGGTGAG) and 16SR (5'-CGCGGCTGCTGGCACGGAGTTAGC) were designed based on the published ribosomal RNA sequences for *Photorhabdus* spp to amplify the partial 16S ribosomal RNA (16S rRNA) sequence which contains several variable regions. PCR products were purified with the QIAquickPCR purification Kit (Qiagen) and directly sequenced by using automated 3730 DNA Analyzer from Applied Biosystems, Inc. and BigDye Terminator Cycle Sequencing Chemistry at the Plant-Microbe Genomics Facility (Ohio State University, Columbus, OH, USA). Previously published 16S rRNA sequences in the GenBank of NCBI from members of family Enterobacteriaceae were used as references with which to compare the sequence of our bacterial isolate (Table 4.1). Sequence alignments and phylogenetic analysis was based on trimmed clean sequences using the program ClustalX1.8 and Mega3.1. Bootstrap analysis was used to evaluate the tree topology by performing 500 resamplings.

**Phenotypic characterization.** Cultural properties such as colony size, shape and color were observed after 72 h incubation at 28°C on BHI agar. Physiological and biochemical tests were performed at 28°C using API20E (BioMerieux, Inc. Durham, NC) in our lab.
and using Sensititre GNID plate done by Ohio Department of Agriculture (Animal Disease Diagnostic Laboratory, Reynoldsburg, OH). The oxidase and motility tests were conducted separately. The oxidase test which determines the presence of cytochrome oxidase is one of the first biochemical tests performed on Gram negative organisms. To perform the oxidase test, a drop of freshly prepared oxidase reagent (1% tetra methyl-para-phenylene diamine dihydrochloride) was placed on a piece of filter paper in a Petri dish (Tarrand & Groschel, 1982). After 1 min, a small amount of the test colony was

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<th>Source</th>
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Table 4.1: *Photorhabdus temperata* isolates, their geographic origins and nematode hosts
rubbed by a wooden stick onto the moistened paper. The positive test was indicated by the rapid appearance of a purple color. The bacterial motility was tested by semi-solid motility test medium (per 1L distilled water: 37g BHI, 3g agar, and 0.04g 2,3,5-triphenyltetrazolium chloride) (Power, 1988). The bacterial motility was recorded as diffusing in the semi-solid motility test medium (containing 0.3% agar and 0.004% TTC). The bacterial resistance to antibiotics was tested on medium plates containing single antibiotics, including ampicillin, kanamycin, streptomycin, carbenicillin, and rifampicin. For each antibiotic, three replicates were used.

4.4 RESULTS AND DISCUSSION

The bacterial isolate from *H. bacteriophora* GPS11 was Gram negative, oxidase negative and catalase positive. This isolate grew well on all the media tested in this study. The colony morphology was granulated, convex, and opaque, and colonies had a sticky consistency. The 48 h cultured single colony is small to medium sized, about 1.0 mm in diameter. This bacterium produced luminescence that was observed in the dark. The colonies were greenish with redish-brown center on NBTA indicator plates, red on MacConkey agar plates after 48 h incubation, and light orange on LB, Nutrient, and BHI plates after 96 h incubation.

PCR amplification of 16S rRNA gene produced a single band of about 1,500 bp, and the validity of the isolate was confirmed based on the comparison of 16S rRNA gene sequence with other *Photorhabdus* species. Although using full 16S rRNA sequences in phylogenetic analysis is limited because the differences in *Photorhabdus* 16S rRNA
sequence are restricted to a few variable regions (Akhurst et al., 2004), partial variable 16S rRNA sequences can be sufficient to establish phylogenetic relationships (Lane et al., 1985; Liu et al., 1997; Yong et al., 1991). Thus, we established the phylogenies based on the partial 16S rRNA sequences that contain several variable regions in this study. As a reference for future studies, when more Photorhabdus isolates become available, the partial 16S rRNA sequences used for phylogenic analysis in this study were flanked by the sequences 5'-CCACTGGAAACGGTGGCTAATACC and 5'-CGCGGCTGCTGGCACGGAG. The 16S rRNA sequences of other P. temperata isolates were from the public database in National Center for Biotechnology Information (NCBI) (Table 4.1). The alignments of the clean partial 16S rRNA sequences comprise approximately 360bp. The partial 16S rRNA sequence of the GPS11 isolate showed 96% similarity to the type strain of P. temperata and 93%-96% similarity to other Photorhabdus species. The 16S rRNA differences between GPS11 isolate and other Photorhabdus species were confined to four regions with inserted nucleotides (Figure 4.1). In order to make sure the insertion was not a false reading, the sample was sequenced twice in different facilities. In the phylogenetic tree of P. temperata built in this study (Figure 4.2), the isolates H4, HL81, Xinch, HSH2, and K122 associated with Heterorhabditis from Europe grouped together, and this is in agreement with the previous study from Fischer-Le Saux et al. (1999) which indicated that these isolates belonged to one subgroup. Isolates Meg, wx6, wx8, wx12, OH1, and P7 associated with either H. meigidis or unidentified Heterorhabditis spp from North America grouped together. The GPS11 isolate grouped together with isolates C1, NC19 and Heliothidis which are
associated with *H. bacteriophora* from North America and isolate Meg1 which is associated with *H. megidis* from North America, as well as with isolates wx11, wx9, wx9hyper, and wx10 associated with unidentified *Heterorhabditis* spp from North America.

In order to test whether the new isolate was identical to *P. temperata* in physiological traits, GNID, API 20E and several other tests were performed (Tables 4.2 and 4.3). All isolates including GPS11 were negative for the following tests: hydrogen sulfide production, activities of gelatinase, catalase, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, fermentation of sobitol, rhamnose, melibiose, arabinose, cellobiose, raffinose, arabitol, sucrose, and xylose, carbon utilization of malonate, lysine, arginine, and ornithine. All isolates produced acid from glucose. Several distinctive characters were observed for the GPS11 isolate. The GPS11 isolate did not produce acid from fructose and maltose. Aesculin hydrolysis is positive only for isolates GPS11 and NC19 which allows these isolates to be distinguished from other isolates. The GPS11 isolate can use citrate as the carbon source which was not used by most other *P. temperata* isolates. The GPS11 isolate is capable of motility in the semi-solid motility test medium and exhibits strong resistance to ampycillin, but it is susceptible to carbenicillin.

Based on 16S rRNA and phenotypic data, we propose to place the GPS11 isolate in a new subspecies of *Photorhabdus temperata* subsp. *boemarei* subsp. nov. It has been well known that *H. bacteriophora* harbors *P. luminescens*, and *P. temperata* is associated with *H. megidis* (Boemare, 2002). The only exceptions are the C1, NC19 and heliothidis.
<table>
<thead>
<tr>
<th>P. temperata strains</th>
<th>GPS11</th>
<th>NC19</th>
<th>Meg</th>
<th>Xinch</th>
<th>NZH3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR1: Lysine 7AMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylose /SF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FR3: 4MU Phosphate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose /SF</td>
<td>-</td>
<td>w</td>
<td>+</td>
<td>w</td>
<td>w</td>
</tr>
<tr>
<td>FR5: Proline 7AMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Arabinose /SF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FR7: γ-Glutamine 7AMC</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malonate /CU</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FR12</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Trehalose /SF</td>
<td>-</td>
<td>w</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FR4: 4MU α-D Glucopyranoside</td>
<td>-</td>
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<tr>
<td>Fructose /SF</td>
<td>-</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>w</td>
</tr>
<tr>
<td>Lysine /CU</td>
<td>-</td>
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<tr>
<td>Arginine /CU</td>
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<tr>
<td>Pyruvate /CU</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ornithine /CU</td>
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<tr>
<td>Sucrose /SF</td>
<td>-</td>
<td>-</td>
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<tr>
<td>FR8: 4MU bis-Phosphate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Inositol /SF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Aesculin hydrolysis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Tryptophan Deaminase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FR6: 4MU α-D Galactopyranoside</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Citrate /CU</td>
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<td>-</td>
<td>-</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol /SF</td>
<td>-</td>
<td>-</td>
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<tr>
<td>FR9: 4MU β-D-Glucuronide</td>
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<tr>
<td>Mannitol /SF</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>FR10: 4MU β-D-</td>
<td>-</td>
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<tr>
<td>Árabinol /SF</td>
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<tr>
<td>Cellobiose /SF</td>
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<tr>
<td>Agmatine /CU</td>
<td>-</td>
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**Table 4.2:** *In vitro* diagnosis of sensitive GNID identification plate for isolated bacteria (credited to Ohio Department of Agriculture). All tests for GPS11 isolate were done at 28°C, and results for other isolates were obtained from previously published data (Akhurst et al., 1996; Fischer-Le Saux et al., 1999). FR: Fluorogenic reagent; FR12: 4MU 2-acetamide-2-deoxyglucopyranoside + 4MU α-L-arabinopyranoside; SF: sugar fermentation; CU: carbon utilization; 4MU: 4-methyl umbelliferone; 7AMC: 7-methyl coumarin amide; +/-: positive; -: negative; w: weakly positive.
<table>
<thead>
<tr>
<th>P. temperata strains</th>
<th>GPS11</th>
<th>NC19</th>
<th>Meg</th>
<th>Xinch</th>
<th>NZH3</th>
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<tr>
<td>β-galactosidase</td>
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<td>Arginine dihydrolase</td>
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<tr>
<td>Lysine decarboxylase</td>
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<tr>
<td>Ornithine decarboxylase</td>
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<tr>
<td>Citrate utilization</td>
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<td>-</td>
<td>w</td>
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<tr>
<td>H₂S production</td>
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<td>-</td>
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<td>Urease</td>
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<td>+</td>
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<td>Sorbitol- Fermentation</td>
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<td>Rhamnose- Fermentation</td>
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<td>Melibiose- Fermentation</td>
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<td>Amygdalin- Fermentation</td>
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<td>w</td>
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<td>Arabinose- Fermentation</td>
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<td>Cytochrome oxidase</td>
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<td>Motility</td>
<td>+</td>
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</table>

**Table 4.3**: Biochemical characteristics of isolated bacteria from nematodes by APE 20E (BioMerieux SA, Durham, NC). All tests for GPS11 isolate were done at 28°C, and test results for other isolates were obtained from previously published data (Akhurst et al., 1996; Fischer-Le Saux et al., 1999). +: positive; -: negative; w: weak positive.
strains of *P. temperata* which were found to be associated with *H. bacteriophora* (Fischer-Le Saux et al., 1999; Marokhazi et al., 2003). However, the DNA hybridization between *P. temperata* C1 and other *P. temperata* isolates from Europe was very low (Nielsen & Lubeck, 2002).

Further, the co-speciation between *P. temperata* and *Heterorhabditis* is ambiguous due to the uncertain taxonomy of both the nematode and bacteria. For example, *P. temperata* has been found to be associated with *H. bacteriophora*, *H. megidis*, *H. downesi*, and *H. zealandica*. As suggested by Fischer-Le Saux et al. (1999) further subgroups or subspecies in *P. temperata* are necessary to remove ambiguity in *Photorhabdus-Heterorhabditis* association. However, the previous proposal of Fischer-Le Saux et al. (1999) for the subspecies *P. temperata* subsp. *temperata* was considered illegitimate due to the fact that it was the only proposed subspecies.

(http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=171441) within the species. As a species and subspecies in Bacteriology is an artificial concept, based on 16S rRNA sequences, phenotypic characters, nematode-bacteria co-speciation and geographical origin data, we suggest three subspecies in *P. temperata*: *P. temperata* subsp. *fischerlesauxii* which is associated with either *H. megidis* or *H. downesi* isolated from Europe, *P. temperata* subsp. *boemarei* subsp. nov. including isolates GPS11, C1, NC19, and Heliothidis which are associated with *H. bacteriophora* isolated in North America, and *P. temperata* subsp. *stackebrandtii* subsp. nov. including isolates Meg, P7 and OH1 which are associated with *H. megidis* isolated from North America. The isolates wx9, wx9hyper, and wx10 associated with unidentified *Heterorhabditis* were
classified to be *P. temperata* subsp. *boemarei* subsp. nov., and the isolates wx6, wx8, and wx12 associated with unidentified *Heterorhabditis* were classified to be *P. temperata* subsp. *stackebrandtii* subsp. nov. The Meg1 isolate associated with *H. megidis* from North America was grouped with *P. temperata* subsp. *boemarei* subsp. nov. in this study, suggesting that re-identification of this nematode strain may be needed in the future. The strain of *P. temperata* associated with *H. zealandica* isolated from the United States was not included in this classification because of the lack of its 16S rRNA sequence at this time. However, based on its phenotypic characters (Tables 4.2 and 4.3), the *H. zealandica* bacterial isolate possibly belongs to another subgroup or subspecies, and further study is needed.

4.5 DESCRIPTION OF PHOTORAHBDUS TEMPERATA SUBSPECIES

_**Description of Photorhabdus temperata subsp. boemarei subsp. nov.**_

*Photorhabdus temperata* subsp. *boemarei* (boe.ma'rei. N.L. gen. n. *boemarei* of Boemare; this sub-species is named after Dr. Noël Boemare in recognition of his contributions to Bacteriology).

Maximum temperature for growth in nutrient broth is 35°C. Cells are Gram-negative, motile, oxidase negative, and catalase, arginine dihydrolase, and gelatinase positive. Compared to other subspecies, the distinct character for *P. temperata* subsp. *boemarei* is arginine dihydrolase positive. The colonies are bioluminescent, granulated, convex, and opaque, and have a sticky consistency. Cells are negative for indole and H$_2$S
production, lysine and ornithine decarboxylases, and β-galactosidase. Acid is produced from inositol and glucose. The cells are variable for urease, tryptophan deaminase, citrate utilization, pyruvate utilization, and aesculin hydrolysis. Isolates are obtained from *H. bacteriophora* species in North America. The type strain is ATCC 29304^T^ which is associated with *H. bacteriophora* NC19. The GenBank accession numbers of the 16S rRNA gene sequences of the type strain is AY278657.

**Description of Photorhabdus temperata subsp. fischerlesauxii subsp. nov.**

*Photorhabdus temperata* subsp. *fischerlesauxii* (fi.scher-le'sau.xii. N.L. gen. n. *fischerlesauxii* of Fischer-Le Saux; this sub-species is named after Dr. Marion Fischer-Le Sauxi who demonstrated the need for subspecies classification in *P. temperata*).

Maximum temperature for growth is 34°C. Cells are Gram-negative, motile, oxidase and arginine dehydrolase negative, and catalase and gelatinase positive. The colonies are bioluminescent. Cells are negative for indole and H$_2$S production, lysine and ornithine decarboxylases, and β-galactosidase. Acid is produced from inositol and glucose, and weakly produced from maltose and fructose. Compared to other subspecies, acid production from trehalose is the distinct character for *P. temperata* subsp. *fischerlesauxii*. They are positive for pyruvate utilization and aesculin hydrolysis, and negative for urease and tryptophan deaminase, and show weak citrate utilization. Isolates are obtained from either *H. megidis* or *H. downesi* in Europe. The type strain is CIP 105563^T^ which is associated with *H. megidis* Xinch, and the GenBank accession number of the 16S rRNA gene sequence of the type strain is AJ007405.
Description of *Photorhabdus temperata* subsp. *stackebrandtii* subsp. nov.

*Photorhabdus temperata* subsp. *stackebrandtii* (sta.cke.brandtii. N.L. gen. n. *stackebrandtii* of Stackebrandt; this subspecies is named after Dr. Erko Stackebrandt in recognition of his contributions to Bacteriology)

Maximum temperature for growth is 34 °C. Cells are Gram-negative, motile, oxidase and arginine dehydrolase negative, and catalase and gelatinase positive. The colonies are bioluminescent. Cells are negative for indole and H₂S production, lysine and ornithine decarboxylases, and β-galactosidase. Acid is produced from inositol, maltose, and glucose, and weakly produced from fructose. Compared to other subspecies, acid production from maltose is the distinct character for *P. temperata* subsp. *stackebrandtii*. They are positive for urease, tryptophan deaminase, and aesculin hydrolysis, and negative for citrate utilization. Isolates are obtained from *H. megidis* in North America. The type strain is proposed to be Meg isolate which should be deposited in the international public collections. The GenBank accession number of the 16S rRNA gene sequence of the type strain is Z76750.

### 4.6 REFERENCES


Figure 4.1: Alignment of partial 16S rRNA sequences between bacterial isolate from *H. bacteriophora* GPS11 and other *Photorhabdus* species. Pit: bacterial isolate; Pt1-Pt5: *P. temperata* species; Pl1 and Pl2: *P. luminescens* species; Pa1 and Pa2: *P. asymbiotica* species.
Figure 4.2: Phylogram obtained by the distance substitution analysis of partial 16S rDNA gene sequences, showing the relationships among *P. temperata* isolates. The tree was constructed by Neighbor-Joining methods with bootstrap analysis of 500 replicates. Numbers at branch-points indicate the number of times (expressed as a percentage) that the sequences to the right of the branch-point were recovered as a monophyletic group in the bootstrap analysis. The names for *P. temperata* isolates are according to the Table 4.1. The scale bar indicates substitution per nucleotide position.
CHAPTER 5

DIFFERENCES IN SUSCEPTIBILITY OF WHITE GRUB SPECIES TO ENTOMOPATHOGENIC NEMATODE SPECIES: THE RELATIVE CONTRIBUTION OF SYMBIOTIC BACTERIA AND NEMATODES

5.1 ABSTRACT

As susceptibility of white grub species to entomopathogenic nematodes differs, we compared the virulence of *Photorhabdus temperata* and *Xenorhabdus koppenhoeferi*, the symbionts of nematodes *Heterorhabditis bacteriophora* and *Steinernema scarabaei*, respectively, to three white grub species *Popillia japonica*, *Rhizotrogus majalis*, and *Cyclocephala borealis*. Both bacteria were virulent to all three grub species even at as low as 2 cells /grub. However, the median lethal dose at 48 h post injection and median lethal time at 20 cells /grub showed that *P. temperata* was more virulent than *X. koppenhoeferi* to *C. borealis*. There were no differences in virulence of two bacteria against *P. japonica* and *R. majalis*. Although *H.*
Bacteriophora carrying *P. temperata* is less pathogenic than *S. scarabaei* carrying *X. koppenhoeferi* to *R. majalis*, *P. temperata* grew faster than *X. koppenhoeferi* both in *vitro* and in *vivo*. We then tested the pathogenicity of oral and hemolymph introduced *H. bacteriophora* to *R. majalis* to determine whether nematodes are able to successfully vector the bacteria into the hemolymph. Hemolymph injected *H. bacteriophora* were pathogenic to *R. majalis* indicating successful bacterial release, but orally introduced *H. bacteriophora* were not. Dissection of grubs confirmed that oral introduced *H. bacteriophora* were unable to penetrate into the hemolymph through the gut wall. Therefore, we conclude that the low susceptibility of *R. majalis* to *H. bacteriophora* is not due to the symbiotic bacteria, but is due to the nematode’s poor ability to penetrate through either the gut wall or the cuticle to vector the bacteria into the hemolymph.

### 5.2 INTRODUCTION

The bacteria *Xenorhabdus* and *Photorhabdus* are symbiotically associated with the entomopathogenic nematodes (EPNs), Steinernematidae and Heterorhabditidae (Boemare, 2002). Naturally, bacteria colonize the gut of nematode infective juveniles (IJs) which represent a specialized stage of development designed for survival in the unfavorable environment. The IJs actively seek out and penetrate potential insect larval hosts and release the bacteria into the insect haemolymph where the bacteria multiply and secrete a wide range of extracellular hydrolytic enzymes that serve to convert the organs and tissues of the insect into an ideal niche for nematode growth and development (Joyce et al., 2006). The insect dies of septicaemia within 24-48 h after nematode infection, and the IJs recover into feeding stage. The
nematodes feed on symbiotic bacteria completing 1-3 generations in the host cadaver, and as food resources are depleted new IJs are produced which reassociate with the symbiotic bacteria and disperse in search of new hosts (Poinar, 1990).

The EPNs have emerged as excellent biological control agents of insect pests (Grewal et al., 2005a). White grubs (Coleoptera: Scarabaeidae) pose significant problems in urban landscapes, ornamental nurseries and fruit crops (Potter, 1998). Japanese beetle (*Popillia japonica*), oriental beetle (*Anomala orientalis*), European chafer (*Rhizotrogus majalis*), and Asiatic garden beetle (*Maladera castanea*) are among the most economically important white grub species in North America (Potter, 1998). While EPNs can be effective biological insecticides for controlling white grubs in turfgrass (Grewal et al., 2005b) or potted nursery stock (Van Tol & Raupp, 2005), their field use is still limited because of the differences in the susceptibility of white grub species to different species of EPNs. Grewal et al. (2002) revealed that the GPS11 strain of *Heterorhabditis bacteriophora* was the most pathogenic strain among the 22 strains of *Heterohabditis* and *Steinernema* tested against *P. japonica*, *A. orientalis*, and *Cyclocephala borealis*, but had low pathogenicity towards *R. majalis*. Another species *Steinernema scarabaei* was found to be more pathogenic than *H. bacteriophora* to *R. majalis* and *P. japonica* (Cappaert & Koppenhofer, 2003; Koppenhofer et al., 2004), but not to *C. borealis* (Koppenhofer et al., 2007).

As symbiotic bacteria play a key role in the virulence of EPNs, the objective of this study was to determine if the different pathogenicity between *H. bacteriophora* and *S. scarabaei* against white grub species is due to the difference in the virulence of symbiotic bacteria or nematodes. First, the virulence of symbiotic bacteria was examined by injecting different numbers of bacterial cells into the grub’s hemolymph.
and recording grub mortality after 24, 48, 72 and 96 h. We then investigated the pathogenicity of the nematode *H. bacteriophora* to *R. majalis* by directly introducing the infective juveniles into the mouth or hemolymph of the third instar grubs.

### 5.3 MATERIALS AND METHODS

**Source of nematodes, white grubs and bacteria**

The infective juveniles of *H. bacteriophora* GPS11 were obtained from our liquid nitrogen frozen stock, and of *S. scarabaei* AMK001 were obtained from Dr. Koppenhöfer (Rutgers University, New Brunswick, New Jersey). *R. majalis* were collected from the Sunleaf Nursery (Madison, Ohio), *C. borealis* from Pleasant Hill Golf Course (Perrysville, Ohio), and *P. japonica* were collected from the OARDC turfgrass lawns (Wooster, Ohio) and Pleasant Hill Golf Course. The field collected grubs were kept at 20°C for 10 days, and only healthy, actively moving grubs were used in all experiments. We isolated bacteria from the infective juveniles of both nematode species following the methods described by Akhurst (Akhurst, 1980). The symbiotic bacterium isolated from *S. scarabaei* AMK001 was confirmed to be *Xenorhabdus koppenhoeferi* AMK001 by the 16S rRNA sequence. The bacterial isolate from *H. bacteriophora* GPS11 was identified to be *Photorhabdus temperata* GPS11 (An and Grewal, unpublished data).

**Pathogenicity of the symbiotic bacteria**

The bacteria *X. koppenhoeferi* AMK001 and *P. temperata* GPS11 were grown on the BHI agar plates for 48 h. The individual colonies were picked and inoculated into 5 ml BHI broth at 28°C until they reached an OD 600nm of 0.8 to 1.0 (1 cm path-
length). Dilutions containing 20,000, 2,000, 200, 20, 2 cells per 10µl for each bacterial species were made using sterile BHI broth. Thirty six grubs each of *P. japonica*, *C. borealis*, and *R. majalis* were injected with 10µl of each dilution from the foreleg by a 33-gauge hypodermic needle. Grubs were surface-sterilized before injection by dipping in 0.1% Thimerosal solution for 10 min and rinsing in sterile distilled water three times. After injection, each grub was placed on 3g autoclaved soil with 100µl sterile water in a well of a 24-well plate (Corning Inc., Corning, NY) and mortality was recorded at 24 h intervals until 96 h at 20°C. There were three types of controls: 36 grubs injected with 10µl of sterile BHI broth, 36 grubs pierced with a needle alone, and 36 uninjected grubs. The experiment was repeated once. Grub mortality data were arcsine transformed and subjected to one-way analysis of variance using the statistical software STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma). The median lethal dose (LD$_{50}$) and the median lethal time (LT$_{50}$) were calculated by Minitab software. Based on the slug mortality data, LD$_{50}$ was calculated by the mortality of grub at 48 h post bacterial injection using regression - Logit fit analysis and LT$_{50}$ was calculated by the mortality of grub with an injection dose of 20 cells using regression – linear fit analysis.

**Growth rates of isolated bacteria**

The growth rates of bacteria *X. koppenhoeferi* AMK001 and *P. temperata* GPS11 in the BHI broth at 28°C were determined by optical density measurements according to Tortora et al. (2004). The growth rates of the two bacterial species were also compared in *R. majalis* by counting the numbers of viable bacteria 48 h post injection. The overnight cultured bacteria were adjusted to a concentration of 20-
cells/10μl, and injected into the hemolymph of the third instar *R. majalis* grubs. The injected grubs were incubated at 20°C, and the numbers of viable bacteria in each grub were determined at 24 h intervals up to 96 h using the following procedure. After incubation, the grubs were surface sterilized by immersing in 0.1% Thimerosal for 3 h, ground using an autoclaved mortar and the grub homogenate was serially (10-fold) diluted and plated on BHI agar. Three replicates were prepared for each time point. All of BHI agar plates were incubated at 28°C for 3 days. Numbers of colony forming units (CFU) were then counted. The experiment was repeated once.

**Pathogenicity of *H. bacteriophora* GPS11 to *R. majalis***

The virulence of *H. bacteriophora* GPS11 to *R. majalis* was examined by direct injection of IJs into the mouth or hemolymph of the third instar grub. Five surface-sterilized IJs with 10μl sterile water were injected into the mouth or hemolymph of *R. majalis*. The oral injection was conducted using a gel loading plastic tip to avoid injury. The nematode was released into the mouth by gently opening the grub mouth with the tip while holding the grub with the thumb and the first finger. The hemolymph injection was conducted by injecting the IJs from the forleg of the grub as described previously. After injection, each grub was placed on 3g autoclaved soil with 100μl sterile water in a well of the 24-well plate and mortality was recorded daily for 96h at 20°C. There were 6 grubs per treatment in each of three replications, and the experiment was repeated once. There were two types of controls: 18 grubs injected with 10μl of sterile water and 18 untreated grubs. Grub mortality data were arcsine transformed and subjected to one-way analysis of variance using STATISTICA 7.0 software.
In a parallel experiment we determined if orally injected nematodes entered into the hemolymph or not. Twelve *R. majalis* were orally injected with 5 surface-sterilized IJs of *H. bacteriophora* GPS11. After oral injection, the grubs were rinsed carefully with tap water to remove any IJs from their exterior, and placed in the 24 well plates. The grubs were then dissected, and the numbers of nematodes in the gut and hemolymph were counted, as well as the numbers of nematodes in the well.

### 5.4 RESULTS

**Virulence of bacteria to white grubs**

Both *X. koppenhoeferi* AMK001 and *P. temperata* GPS11 were pathogenic to all three species of white grubs when injected into the hemolymph (Figure 5.1-5.3). The grub mortality reached almost 100% in all treatments at 72 h post injection, and over 50% at 48 h post injection with a dose of 200 cells or higher per grub. The LD$_{50}$ value at 48 h post injection for *P. temperata* GPS11 and *X. koppenhoeferi* AMK001 against *C. borealis* differed significantly, but not against *P. japonica* or *R. majalis* (Table 5.1), suggesting that *C. borealis* is more susceptible to *P. temperata* GPS11 than to *X. koppenhoeferi* AMK001. The LT$_{50}$ value at the injection dose of 20 cells per grub also showed significant difference between *P. temperata* GPS11 and *X. koppenhoeferi* AMK001 against *C. borealis* (Table 5.1), but not against *P. japonica* or *R. majalis*, suggesting that *C. borealis* is killed sooner by *P. temperata* GPS11 than by *X. koppenhoeferi* AMK001.

**Bacterial growth rates**

In culture, both bacteria showed classic sigmoid growth curve, except that the
Table 5.1: The median lethal dose (LD₅₀ in colony forming units, N=24) of Photorhabdus temperata GPS11 and Xenorhabdus koppenhoeferi AMK001 against Popillia japonica, Rhizotrogus majalis, and Cyclocephala borealis at 48 h post injection and the median lethal time (LT₅₀ in h, N=18) with an injection dose of 20 cells per grub.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>X. koppenhoeferi AMK001</th>
<th>P. temperata GPS11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grubs</td>
<td>P. japonica</td>
<td>R. majalis</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>102</td>
<td>29</td>
</tr>
<tr>
<td>Slope</td>
<td>0.91</td>
<td>0.83</td>
</tr>
<tr>
<td>95%CI</td>
<td>72-153</td>
<td>16-48</td>
</tr>
<tr>
<td>LT₅₀</td>
<td>54</td>
<td>50</td>
</tr>
<tr>
<td>Slope</td>
<td>0.87</td>
<td>0.98</td>
</tr>
<tr>
<td>95%CI</td>
<td>50-59</td>
<td>48-50</td>
</tr>
</tbody>
</table>

time to enter log phase and stationary phase was faster for P. temperata GPS11 than X. koppenhoeferi AMK001 (Figure 5.4). It took 20 h for P. temperata GPS11 and 40 h for X. koppenhoeferi AMK001 to enter log phase, and 40 h for P. temperata GPS11 and 60 h for X. koppenhoeferi AMK001 to enter stationary phase. Under in vivo conditions in R. majalis (Figure 5.4), P. temperata GPS11 also grew faster than X. koppenhoeferi AMK001 reaching exponential phase within 48 h post injection.

Pathogenicity of H. bacteriophora injected into the mouth or hemolymph of R. majalis

There were significant differences (P < 0.05) in the pathogenicity of H. bacteriophora to R. majalis between oral and hemolymph injections (Figure 5.5). While the nematode injected into the hemolymph caused significantly higher
mortality of *R. majalis* compared to the control, the nematode introduced into the mouth did not. Dissection of the orally injected grubs showed that the nematodes did not persist in the gut or enter into hemolymph after 48h, but were defecated into the well. Thus, the penetration of the nematode into the hemolymph was not successful when the nematodes were released into the gut via oral injection.

### 5.5 DISCUSSION

Our study showed that bacteria *P. temperata* GPS11 and *X. koppenhoeferi* AMK001 injected into the hemolymph were both highly virulent to all three white grub species *P. japonica*, *R. majalis*, and *C. borealis*. These results are consistent with previous studies testing the virulence of *Photorhabdus* sp. to different insects. When injected into the haemolymph, *P. temperata* K122 was highly virulent to *Galleria mellonella* (Clarke & Dowds, 1995; Watson *et al.*., 2005) and *Photorhabdus luminescens* W14 was highly virulent to *Manduca sexta* (Daborn *et al*., 2001). Since the symbiotic bacteria *X. koppenhoeferi* AMK001 and *P. temperata* GPS11 were both extremely virulent with LD$_{50}$ value around 29-35 cfu per grub at 48 h post injection, we conclude that the poor pathogenicity of *H. bacteriophora* GPS11 to *R. majalis* was not due to its symbiotic bacteria.

Besides the high mortality caused by *P. temperata* GPS11 and *X. koppenhoeferi* AMK001, the two bacteria showed differences in virulence to *C. borealis*. The LD$_{50}$ at 48 h post infection and LT$_{50}$ at the injection dose of 20 cells per grub indicated that *P. temperata* GPS11 was significantly more virulent to *C. borealis* than *X. koppenhoeferi* AMK001. This may be due to the more rapid growth rate of *P. temperata* compared to *X. koppenhoeferi* at the tested temperature. It has also been
shown that virulence of *P. luminescens* to other insects is tightly correlated with bacterial growth rate (Clarke & Dowds, 1995; Daborn *et al.*, 2001; Joyce *et al.*, 2006; Watson *et al.*, 2005). In addition, the higher virulence of *P. temperata* to *C. borealis* may be partially attributed to the fact that *P. temperata* GPS11 associated nematode *H. bacteriophora* GPS11 was originally isolated from *C. borealis* (Grewal *et al.*, 2002). Although the growth rate of *P. temperata* GPS11 was faster than *X. koppenhoeferi* AMK001 in *R. majalis*, the LT<sub>50</sub> and LD<sub>50</sub> values showed no differences in the virulence of two bacteria to either *R. majalis* or *P. japonica*. This may suggest that the two bacteria use different virulence mechanisms to kill *R. majalis* and *P. japonica*.

Vectoring symbiotic bacteria into the insect hemolymph is one of the most important steps for successful infection of the insect by EPNs. Nematode penetration into the hemocoel can occur directly through the cuticle, midgut epithelium, or tracheae via the spiracles in different insect species. In white grubs, the spiracle route is not accessible to nematodes because spiracles are covered with sieve plates impenetrable to the nematodes (Forschler & Gardner, 1991; Galbreath, 1976; Hinton, 1967). Our results showed that *H. bacteriophora* GPS11 introduced into the hemolymph with 5 IJs was highly virulent to *R. majalis* compared to the control. This suggests that the insect immune response was not effective to kill the invading nematodes or prevent them from releasing bacteria. Considering that each IJ of *H. bacteriophora* GPS11 carries about 10 – 30 *P. temperata* GPS11 cells (An and Grewal, unpublished data), the injection of 5 IJs/ grub would have resulted in the release of about 50 -150 bacterial cells/ grub. However, comparing the grub mortality pattern caused by nematode injection with that caused by bacteria injection, the pattern caused by nematode injection was similar to that caused by an injection of
20 bacterial cells per grub. Thus, we conclude that upon nematode injection, only a small portion of bacteria carried by nematode IJs were released into hemolymph within 24 h.

As a small dose of *H. bacteriophora GPS11* was highly virulent to *R. majalis* when introduced into the hemolymph via injection, penetration of *H. bacteriophora GPS11* into *R. majalis* was thus not successful in nature. Although both *Heterorhabditis zealandica* and *H. bacteriophora* showed excellent cuticular penetration ability (Koppenhofer et al., 2007), *R. majalis* mortality increased significantly with exposure time for *H. zealandica* but not for *H. bacteriophora*. This suggests that the cuticular penetration of *H. bacteriophora* into *R. majalis* can not be enhanced by elongated expose time, and thus the cuticular penetration is not successful. Also the proposed poor ability of *H. bacteriophora* penetrating into *R. majalis* through cuticle could be supported by the fact that despite the high number of IJs exposed to the white grubs tested in the laboratory, the infectivity of *H. bacteriophora* to *R. majalis* was still very low (Grewal et al., 2002; Koppenhofer et al., 2004; Koppenhofer et al., 2006; Koppenhofer et al., 2007).

*H. bacteriophora* commonly penetrate through the cuticle but may possess gut penetration ability (Koppenhofer et al., 2007). Penetration through the midgut epithelium into the hemolymph involves a probing behavior which is stimulated by the gut fluid (Grewal et al., 1993). However, nematode penetration through the midgut epithelium into the hemolymph is often delayed by the peritrophic membrane (Cui et al., 1993; Forschler & Gardner, 1991; Thurston et al., 1994), and this delay increases the possibility of removal of the nematodes by repellence of the insect feces (Thurston et al., 1994). Our results showed that the infectivity of *H. bacteriophora* to
R. majalis by oral injection was poor and IJs were unable to penetrate into the hemolymph through the gut wall and were removed into the environment within 48 h. These results are consistent with a previous study for H. bacteriophora HP88 strain which showed that the infectivity of H. bacteriophora HP88 strain to P. japonica could not be enhanced by grub feeding (Wang & Gaugler, 1998), and the rate of penetration from gut to hemolymph after oral injection was only about 1.3% regardless of injection dose. Combining these data with our results, therefore, we conclude that the low susceptibility of R. majalis to H. bacteriophora is also due to nematode’s poor ability to penetrate through the gut wall to vector the bacteria into grub’s hemolymph.

The results and discussion presented above indicate that the unsuccessful penetration of H. bacteriophora GPS11 into the hemolymph is the main reason for low pathogenicity of this nematode to R. majalis. This study also indicates the importance of the tripartite interactions among bacteria, nematodes, and insect for successful infection. Therefore, future research may focus on how to improve the penetration ability of H. bacteriophora GPS11.

5.6 REFERENCES


Figure 5.1: The mortality of grub *Popillia japonica* at 48 h (a) and 72 h (b) post bacterial infection via hemolymph injection.
Figure 5.2: The mortality of grub *Rhizotrogus majalis* at 48 h (a) and 72 h (b) post bacterial infection via hemolymph injection.
Figure 5.3: The mortality of grub *Cyclocephala borealis* at 48 h (a) and 72 h (b) post bacterial infection via hemolymph injection.
Figure 5.4: Growth curves for *X. koppenhoeferi* AMK001 and *P. temperata* GPS11.
(a) Growth curve in culture derived from measuring OD600 overtime. (b) Bacterial growth curve during a grub infection derived from the mean number of CFU recovered from infected grubs at different time points post bacterial injection.
Figure 5.5: The mortality of grub *R. majalis* after injection of 5 infective juvenile *H. bacteriophora* GPS11 from mouth (Nematode-M) or hemolymph (Nematode-H). The injected water served as the control.
CHAPTER 6

GENE EXPRESSION OF *PHOTORHABDUS TEMPERATA* AND *XENORHABDUS KOPPENHOEFERI* DURING INFECTION TO THE WHITE GRUB *RHIZOTROGUS MAJALIS*

6.1 ABSTRACT

Pathogenicity of the entomopathogenic nematodes to their grub hosts are primarily determined by the nematode symbiotic bacteria. Inasmuch as the susceptibility of white grub species to the nematode-bacteria symbiotic complex differs, we compared gene expression of bacteria *Photorhabdus temperata* (symbiont of *Heterorhabditis bacteriophora*) and *Xenorhabdus koppenhoeferi* (symbiont of *Steinernema scarabaei*) during infection to the white grub *Rhizotrogus majalis*. We identified 30 genes by *P. temperata* and 25 by *X. koppenhoeferi* differentially expressed at 24 h post bacterial
injection using selective capture of transcribed sequences (SCOTS) technique. These genes could be divided into seven major functional groups including nutrient scavenging, virulence, stress response, cell surface structure, metabolism, and regulation. Genes commonly up-regulated by both bacteria included $tcaC$ encoding a toxin complex, $surA$ and $uspB$ encoding stress proteins, $accA$ and $thiA$ encoding fatty acid synthesis proteins, $omp$ encoding outer membrane proteins, $hemS$ and $phlA$ corresponding to hemolysin, $gluS$ and $gluR$ corresponding to glutathione, $dsb$ gene family, ubiquinone related genes, and $phoP$ encoding two component system. Other up-regulated genes were unique to either $P.\ temperata$ or $X.\ koppenhoeferi$, in which $ttsL$ encoding a type III secretion system was only identified from $P.\ temperata$, and $lpsE$ corresponding to lipopolysaccharide synthesis was unique to $X.\ koppenhoeferi$. A metabolic computational pathway analysis for the differentially expressed genes revealed that a total of 23 of 30 up-regulated genes from $P.\ temperata$ and 20 out of 25 from $X.\ koppenhoeferi$ were a part of dense protein networks showing multiple connectivity, suggesting that these genes may act in concert to achieve successful infection. These network genes fell into 16 and 13 general cell processes for $P.\ temperata$ and $X.\ koppenhoeferi$, respectively. Five nexus genes directly connecting to several other genes in the network are identified as attractive candidates for follow-up studies.

### 6.2 INTRODUCTION

Entomopathogenic nematodes (EPNs), Heterorhabditidae and Steinernematidae, have potential for the biological control of many insect pests (Grewal et al., 2005a).
These nematodes are symbiotically associated with bacteria in the family Enterobacteriaceae which are pathogenic to insects: *Heterorhabditis* are associated with *Photorhabdus* and *Steinernema* with *Xenorhabdus* bacteria (Boemare, 2002). In nature, bacteria colonize the gut of the infective juvenile nematode. The infective juveniles (IJs) represent a specialized stage of development suitable for survival and dispersal. The IJs actively seek out and penetrate potential insect hosts and release the bacteria into the insect haemolymph where the bacteria are expected to replicate and secrete a wide range of toxins and extracellular hydrolytic enzymes that serve to convert the organs and tissues of the insect into an ideal niche for nematode growth and development (Joyce *et al.*, 2006). Finally, the insect dies, and the IJs recover into fourth stage juveniles which feed on symbiotic bacteria completing 1-3 generations in the host cadaver. As food resources are depleted new IJs are produced which re-associate with the symbiotic bacteria and disperse in search of new hosts (Poinar, 1990).

Studies have indicated that *Photorhabdus* and *Xenorhabdus* produce toxins to kill the host insects (ffrench-Constant *et al.*, 2003). One toxin complex (Tc) has been identified from both *Photorhabdus luminescens* and *Xenorhabdus nematophila* (Bowen *et al.*, 1998; fffrench-Constant & Bowen, 1999). A functional type III secretion system has been identified in *P. luminescens*, and this type III secretion system has been shown to secrete LopT, a homologue of YopT effector protein from *Yersinia pestis* (Brugirard-Ricaud *et al.*, 2004; 2005). It was recently shown that a mutation in *phoP*, encoding a two component system, rendered *P. luminescens* avirulent to insect larvae (Derzelle *et al.*, 2004). Further, the genome sequence of *P. luminescens* TT01 strain indicates the
presence of the pathogenicity islands (Duchaud et al., 2003; Waterfield et al., 2002) such as Mcf (makes caterpillars floppy) toxins (Daborn et al., 2002; Waterfield et al., 2001a). Recently, a highly conserved toxin gene \textit{txp40}, encoding a ubiquitous insecticidal toxin protein, was identified from 59 strains of \textit{Xenorhabdus} and \textit{Photorhabdus} bacteria, and this toxin has been shown to be active against a variety of insect species (Brown et al., 2006). Lrp, a leucine-responsive regulatory protein, was found to play important roles in virulence of \textit{X. nematophila} and suppression of insect immunity (Cowles et al., 2007).

Gene \textit{xaxAB} encoding a peptide cytotoxin Xax purified from \textit{X. nematophila} broth growth has been cloned recently, and the expression of this gene in recombinant \textit{Escherichia coli} led to the production of active cytotoxin/hemolysin. Further, \textit{xaxAB} has been shown to be present in various \textit{Xenorahbdus} and \textit{Photorhabdus} bacteria (Vigneux et al., 2007). However, very little is known about how toxin production is regulated, although there is some evidence that \textit{P. luminescens} express Tc toxins during exponential growth in the insect larvae (Daborn et al., 2001).

As pathogenicity of EPNs is governed by both nematodes and their symbiotic bacteria, we evaluated the virulence of \textit{Photorhabdus temperata} (symbiont of EPN \textit{Heterorhabditis bacteriophora} GPS11) and \textit{Xenorhabdus koppenhoeferi} (symbiont of EPN \textit{Steinernema scarabaei}) to white grubs by directly injecting bacteria into the grub haemolymph (Chapter 5). Unlike nematode partners, both bacterial species were highly virulent to \textit{R. majalis}, with LD$_{50}$ values in the range of 29-35 colony-forming units per grub at 48 h post injection (An and Grewal, unpublished data). In addition, although \textit{X. koppenhoeferi} grew slower than \textit{P. temperata} both \textit{in vitro} and \textit{in vivo}, the LD$_{50}$ and LT$_{50}$
at 20 cells /grub did not differ between two bacteria. We hypothesized that the two bacteria may use different virulence factors to kill *R. majalis*, and it would be interesting to demonstrate how the two bacterial genera use different gene products to adapt to the same life history. Therefore, we investigated the gene expression of the two bacterial species during infection to *R. majalis* using selective capture of transcribed sequences (SCOTS) technique originally developed by Graham & Clark-Curtiss (1999).

Furthermore, compared to the extensive literatures on the genes and pathways expressed by pathogenic bacteria in higher order hosts, there are few reports on the bacterial gene expression involved in the relatively primitive host. Additionally, it will be very interesting to identify common themes in bacterial pathogenesis across a variety of host-pathogen interfaces because primitive function in the physiology of bacterial pathogens may be maintained despite extreme plasticity due to evolution, specialization, horizontal gene transfer, and recombination.

### 6.3 MATERIALS AND METHODS

**Bacteria, grubs, and culture conditions**

The symbiotic bacteria were isolated from respective nematodes *Heterorhabditis bacteriophora* GPS11 and *Steinernema scarabaei* (An and Grewal, unpublished data), and they were confirmed to be *Photorhabdus temperata* and *Xenorhabdus koppenhoeferi* by 16S rDNA gene sequences. The bacteria were cultured in Brain Heart Infusion (BHI) (Difco) broth at 28°C. European chafers *Rhizotrogus majalis* were collected from
Sunleaf Nursery (Madison, Ohio). The field collected European chafers were kept at 20°C for 10 days, and only healthy, actively moving grubs were used in the experiments.

**General techniques**

Bacterial genomic DNA was prepared using standard method for Gram negative bacteria (Sambrook et al., 2000). Biotinylation of bacterial genomic DNA was obtained with EZ-Link Psoralen-PEO-Biotin (Pierce) according to the manufacturer’s instructions. The total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were treated with RNase-free DNase I (Ambion, Austin, TX) according to the manufacturer’s guidelines, and were concentrated by spectrophotometer and gel electrophoresis. Total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase (Invitrogen RT-PCR kit) according to the manufacturer’s instructions. First strand cDNA was made double-stranded with Klenow fragment (NEB, Beverly, MA) as described by Froussard (Froussard, 1992).

**SCOTS analysis**

We extended SCOTS technique to determine the *in vivo* gene expression of *P. temperata* and *X. koppenhoeferi* in *R. majalis* post infection. Briefly, for the control, 5μg total RNA obtained from 48 h *P. temperata* or *X. koppenhoeferi* cultures (OD600=0.8) were used to make double-stranded cDNAs using primer SCOT09 (5-ATCCACCTATCCCAGTAGGAGNNNNNNNNN). cDNAs were then PCR amplified using primer SCOT0 (5-ATCCACCTATCCCAGTAGGAG) for 30 cycles. Bacterial
cDNA normalization was done as described below. In this study, the ribosomal operons (rDNA) of *P. temperata* and *X. koppenhoeferi* were amplified and used to block the abundant rRNA sequence in order to effectively capture the cDNA molecules representing mRNA transcripts. The rDNA operon was added to biotinylated genomic DNA at a ratio of 10:1. The genomic DNA-rDNA mixture was sonicated to a size range of 1 to 5kb. The sonicated, biotinylated genomic DNA-rDNA mixture containing 6μg rDNA and 0.6μg genomic DNA was denatured and hybridized for 30 min at 65°C. PCR amplified cDNAs (6μg) from 48 h cultures were denatured and added to the genomic DNA-rDNA prehybridized mixture, and hybridized at 65°C for 24 h. Streptavidin magnesphere paramagnetic particles (Invitrogen) were used to capture the bacterial cDNAs that hybridized to biotinylated genomic DNA according to the manufacturer’s instructions. Captured cDNAs were then eluted, precipitated, and amplified by PCR with the primer SCOT0 for additional two successive rounds of SCOTS. The finally amplified cDNAs were considered to be normalized *in vitro* cDNAs. For the treatment, each 10μl suspension of 48 h *P. temperata* or *X. koppenhoeferi* cultures (OD 600 = 0.8) containing 1 x 10^4 cells was inoculated into the hemolymph of *R. majalis* from the foreleg. The total RNA was isolated from the survived grubs at 24 h post injection. Three 5μg total RNA samples obtained from three infected grubs for each bacterium were pooled and made double-stranded by random primers SCOT189 (5'-GACAGATTGCACCTAACCCTNNN). Double-stranded cDNAs were then PCR amplified for 30 cycles by primers SCOT18 (5'-GACAGATTGCACCTAACCCT). Amplified cDNAs were added to the genomic DNA-rRNA prehybridized mixture to
hybridize at 65°C for 24 h. The bacterial cDNAs that hybridized to bacterial genomic DNA were captured by streptavidin-coated magnetic particles. The captured cDNAs were eluted and PCR amplified. In the first round of SCOTS, three separate samples of cDNA were captured by hybridization to biotinylated genomic DNA in parallel reactions. After the first round of SCOTS, the three amplified cDNAs were combined, denatured, and again hybridized to the genomic DNA-rDNA mixture for two successive rounds of SCOTS. The finally amplified cDNA mixtures were considered to be normalized \textit{in vivo} cDNA molecules. To enrich the normalized \textit{in vivo} cDNAs, the normalized cDNAs from each bacterium were hybridized to biotinylated genomic DNA that has been prehybridized with both rDNA and normalized \textit{in vitro} cDNAs. After hybridization, the bacterial cDNAs were captured and PCR amplified for next round of enrichment. Finally, the enriched bacterial cDNAs were cloned into the original TA cloning kit (Invitrogen) to construct the subtractive library.

**Screening individual clones**

Individual cloned inserts in the subtractive library were screened to identify up-regulated genes by dot blot hybridization with probes made from \textit{in vitro} or \textit{in vivo} normalized cDNA mixtures obtained by SCOTS. Probes were digoxigenin-labeled using PCR Dig Probe Synthesis kit from Roche Molecular Biochemicals (Indianapolis, Ind.) in accordance with the manufacturer’s instructions. All individual clones from each subtractive library (approximately 150) were picked to be PCR amplified with the pair of primers M13. Ten micro liter of each amplified product was mixed with 70 µl 20 × SSC
(1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and then 80 µl sample mixture was transferred to each well of dot-blot containing the positively charged nylon membrane using the low vacuum. The nylon membrane with samples was denatured with denature buffer (3M NaCl, 0.4M NaOH) for 10 min at room temperature, and neutralized in 1 × PBS buffer (0.1M NaCl, 7mM Na$_2$HPO$_4$, and 3mM NaH$_2$PO$_4$, pH 6.8) for 10 min at room temperature. The membrane was dried by baking at 80°C for 2 h, and then the baked membrane was rinsed with 2 × SSC and soaked in 2 × SSC for 5 m. The membrane was transferred into a hybridization bottle for hybridization using Dig easy hyb granules (Roche) according to the manufacturer’s instruction. Digoxigenin-labeled cDNA probe mixtures were denatured and added to the hybridization bottles containing the membrane and the hybridization buffer. Hybridization continued at 65°C for approximately 24 h. The membrane was washed briefly with 2 × SSC at room temperature and then twice with 1 × SSC - 0.1% SDS for 15 min each time; these washes were done at 65°C. A final brief rinse with 0.1 × SSC at room temperature completed the washing process. The membrane was incubated at room temperature with 4 ml 1 × SSC with 8% dry milk for 30 m. Four milliliter dilution of anti-digoxigenin-HRP conjugate (1:800) was added, and incubation continues 1 h at room temperature. The membrane was briefly washed as describe above, and successful hybridization was detected by Amersham ECL Plus western blotting detection reagents (GE healthcare Bio-Sciences Corp, Piscataway, NJ) using chemiluminescent detection. The individual clones that only hybridized to the probe made from normalized in vivo cDNAs were chosen for sequence analysis.
Bioinformatics analysis

The selected clones were sequenced at the Biotechnology Center, Madison, WI, USA. These sequences were edited and assembled using EDITSEQ (DNASTAR), ClustalX, and Contig (Vector NTI). Similar sequences were identified using BLAST algorithms (blastx and tblastx) in GenBank of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The functions of identified sequences were assigned using Gene Ontology (www.geneontology.org) and MultiFun (www.ecocyc.org).

Pathway analysis

Since the genes identified in this study are up-regulated during infection to *R. majalis*, there may be some links among these genes. Thus, protein metabolic pathways were inferred from the gene expression data by PathwayStudio 5.0 software (Ariadne Genomics Inc., Rockville, MD) using bacteria database from Kyoto Encyclopedia of Genes and Genomes (KEGG), Database of Interacting Proteins (DIP), and Biomolecular Interaction Network Database (BIND). PathwayStudio program uses natural language scans of PubMed to define connectivity among genes and to delineate a functionally-related network. We assigned ID for each identified genes according to Entrez, and then the list of assigned gene IDs were input into the PathwayStudio program to build the pathway network by finding the shortest path between selected entities. The created
network could demonstrate what cellular processes and pathways the gene products involved in.

6.4 RESULTS

Transcripts expressed by *P. temperata* and *X. koppenhoeferi* within the grub *R. majalis* at 24 h post injection were identified by subjecting the *in vivo* cDNAs to three iterations of SCOTS in the presence of the transcripts expressed by 48 h *in vitro* cultured bacteria. The SCOTS enriched cDNAs in the grub represented differentially expressed genes which were either in lower abundance or absent in 48 h *in vitro* cultures.

**Nucleotide sequence analysis of *in vivo* expressed genes.** A total of 300 colonies from enrichment cDNA libraries were picked for dot blot screening (150 for *P. temperata*, and 150 for *X. koppenhoeferi*). Out of 300 colonies, 100 for *P. temperata* and 75 for *X. koppenhoeferi* were confirmed to be up-regulated transcripts by dot blot hybridization and were sequenced. We identified 30 genes up-regulated by *P. temperata* (Table 6.1) and 25 genes by *X. koppenhoeferi* (Table 6.2) in *R. majalis*. As the duplicate cDNA molecules were detected in the screened library for 90% (49/55) identified genes (Table 6.1 and 6.2), thus, it may predict that libraries of up-regulated genes are redundant and most bacterial up-regulated genes within *R. majalis* have been identified. These genes could be divided into seven functional groups: nutrient scavenging, virulence, stress response, cell surface structure, metabolism, regulation, and uncharacterized genes. Several of these genes were commonly expressed by both bacteria within *R. majalis*. The
common genes were identified not only by sequence similarity but also within the similar pathways in cellular physiology. These common genes include $\text{tcaC}$ encoding toxin complex, $\text{surA}$ and $\text{uspB}$ encoding stress proteins, $\text{phoP}$ encoding a two component system, $\text{accA (thiA)}$ playing a role in acetyl-coA generation, $\text{hemS (phlA)}$ encoding hemolysin related proteins, $\text{dsb}$ gene family ($\text{dsbA}$ and $\text{dsbC}$), $\text{gluS (glur)}$ encoding proteins related to glutathione synthesis, several genes encoding outer membrane proteins, and genes related to ubiquinone synthesis ($\text{ubiM}$ and $\text{nqrD}$). Other genes were unique to each of the two bacteria. For instance, $\text{ttsL}$ encoding a type III secretion system was only identified from $\text{P. temperata}$, and $\text{lpsE}$ corresponding to lipopolysaccharide synthesis was unique to $\text{X. koppenhoeferi}$.

**Pathway network analysis.** When analyzed by PathwayStudio, functional connectivity of gene products identified in this study indicated by the number of lines among object nodes (Figure 6.1 and 6.2). Totally 23 genes out of 30 from $\text{P. temperata}$ and 20 out of 25 from $\text{X. koppenhoeferi}$ were found to be part of dense networks. Multiple connectivity in an indirect manner by the shortest path indicated that these genes may act in concert to achieve successful infection. The genes fell into 16 and 13 general cell processes for $\text{P. temperata}$ and $\text{X. koppenhoeferi}$, respectively. Some genes were found to regulate multiple cell processes. For example, gene $\text{fliI}$ appears to regulate three cell processes: virulence, secretion, and mutagenesis.

Additionally, most commonly up-regulated genes by both bacteria were in the dense network, except genes $\text{omp}$, $\text{accA}$, $\text{uspB}$, and $\text{ubiM}$. Other genes, including $\text{lip}$ and
<table>
<thead>
<tr>
<th>Gene Category</th>
<th>Gene</th>
<th>Clones&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identity&lt;sup&gt;b&lt;/sup&gt;, organism</th>
<th>Putative Gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient</td>
<td>sidB</td>
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<td>75/75, <em>Photorhabdus luminescens</em></td>
<td>Siderophore biosynthesis protein</td>
</tr>
<tr>
<td></td>
<td>sidR</td>
<td>4</td>
<td>74/78, <em>P. luminescens</em></td>
<td>Siderophore receptor</td>
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<tr>
<td></td>
<td>abcA</td>
<td>5</td>
<td>51/53, <em>P. luminescens</em></td>
<td>ABC-binding protein</td>
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<td>Virulence</td>
<td>hemS</td>
<td>6</td>
<td>51/51, <em>P. luminescens</em></td>
<td>Hemolysin secretion protein</td>
</tr>
<tr>
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<td>tcaC</td>
<td>5</td>
<td>46/46, <em>P. luminescens</em></td>
<td>Toxin complex</td>
</tr>
<tr>
<td></td>
<td>ttsL</td>
<td>3</td>
<td>61/61, <em>P. luminescens</em></td>
<td>Type III secretion protein</td>
</tr>
<tr>
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<td>2</td>
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<td>Phospholipase A</td>
</tr>
<tr>
<td></td>
<td>toxC</td>
<td>4</td>
<td>63/63, <em>P. luminescens</em></td>
<td>Putative insectidal toxin</td>
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<tr>
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<td>41/44, <em>P. luminescens</em></td>
<td>Signal peptidase II</td>
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<tr>
<td></td>
<td>virB</td>
<td>4</td>
<td>50/50, <em>P. luminescens</em></td>
<td>Hypothetic virulence protein</td>
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<td>Stress response</td>
<td>gluS</td>
<td>3</td>
<td>83/83, <em>P. luminescens</em></td>
<td>Glutathione synthetase</td>
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<tr>
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<td>surA</td>
<td>5</td>
<td>60/68, <em>P. luminescens</em></td>
<td>Survival protein SurA precursor</td>
</tr>
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<td></td>
<td>uspB</td>
<td>2</td>
<td>49/50, <em>P. luminescens</em></td>
<td>Universal stress protein</td>
</tr>
<tr>
<td></td>
<td>hspC</td>
<td>5</td>
<td>35/35, <em>P. luminescens</em></td>
<td>Heat shock protein</td>
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<tr>
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<td>Aldehyde dehydrogenase</td>
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<td>dshA</td>
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<td>53/64, <em>P. luminescens</em></td>
<td>Disulfide interchange protein</td>
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<td>thiA</td>
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<td>Lipoprotein</td>
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<td>flII</td>
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<td>73/73, <em>P. luminescens</em></td>
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<td>phoP</td>
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<td>73/75, <em>P. luminescens</em></td>
<td>Two-component response protein</td>
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<td>No similarity</td>
<td>Unknown hypothetic protein</td>
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Table 6.1: SCOTS identified *P. temperata* genes differentially expressed in *R. majalis*. "a" Clones means the number of cDNA clones for each identified sequence in the screening library; "b" Identity applies to amino acids obtained from public databases (National Center for Biotechnology Information) with highest similarity to SCOTS sequences.
<table>
<thead>
<tr>
<th>Gene Category</th>
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<th>Identity(^b), organism</th>
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<td>ABC transporter</td>
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<td>Toxin complex</td>
</tr>
<tr>
<td></td>
<td>phlA</td>
<td>2</td>
<td>59/59, <em>Xenorhabdus nematophila</em></td>
<td>Hemolysin protein</td>
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<td></td>
<td>virH</td>
<td>4</td>
<td>62/73, <em>P. luminescens</em></td>
<td>Hypothetical virulence protein</td>
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<td></td>
<td>rtxC</td>
<td>3</td>
<td>53/71, <em>Vibrio vulnificus</em></td>
<td>Lysine acetyltransferase</td>
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<td></td>
<td>resP</td>
<td>4</td>
<td>45/78, <em>P. luminescens</em></td>
<td>Putative resistant protein</td>
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<td>Stress response</td>
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<td>surA</td>
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<td>34/69, <em>Y. pestis</em></td>
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<td></td>
<td>sspA</td>
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<td>56/64, <em>P. luminescens</em></td>
<td>Stringent starvation protein A</td>
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<td>Flagellar M-ring protein</td>
</tr>
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<td>Two-component response protein</td>
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<td>36/51, <em>P. luminescens</em></td>
<td>Disulfide isomerase</td>
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<tr>
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<td>gluR</td>
<td>4</td>
<td>54/59, <em>Y. frederiksenii</em></td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td></td>
<td>ubiM</td>
<td>5</td>
<td>53/63, <em>P. luminescens</em></td>
<td>Demethylubiquinone methyltransferase</td>
</tr>
<tr>
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<td>clpP</td>
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<td>45/57, <em>P. luminescens</em></td>
<td>ATP-dependent protease</td>
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<td></td>
<td>trxB</td>
<td>6</td>
<td>64/82, <em>P. luminescens</em></td>
<td>Thioredoxin reductase</td>
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<td></td>
<td>rpoB</td>
<td>3</td>
<td>58/59, <em>P. luminescens</em></td>
<td>DNA-directed RNA polymerase</td>
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<tr>
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<td>lysC</td>
<td>2</td>
<td>62/74, <em>Y. pestis</em></td>
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<tr>
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<td></td>
<td>3</td>
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<td>Unknown</td>
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</table>

**Table 6.2:** SCOTS identified *X. koppenhoeferi* genes differentially expressed in *R. majalis*. "a" Clones means the number of cDNA clones for each identified sequence in the screening library; "b" Identity applies to amino acids obtained from public databases (National Center for Biotechnology Information) with highest similarity to SCOTS sequences.
four uncharacterized genes from *P. temperata* and *ubiM, lysC*, and two uncharacterized genes from *X. koppenhoeferi*, have not been linked heretofore to other up-regulated genes identified in this study. Because PathwayStudio could not establish links among these genes or to other genes using currently published databases, the pathways they may be involved in are possibly novel.

### 6.5 DISCUSSION

In this study, we have extended the use of SCOTS technique to determine *in vivo* expressed genes from two distinct nematode symbiotic bacteria *P. temperata* and *X. koppenhoeferi* during infection to the invertebrate host *R. majalis*. Notably, our data showed that besides a few common genes, many up-regulated genes were different between the two bacteria during infection to *R. majalis*. Some virulence factors, survival proteins and global regulators common to both *P. temperata* and *X. koppenhoeferi* are similarly up-regulated during infection to *R. majalis*, suggesting the universal virulence mechanism in bacterial pathogens. These commonly expressed genes may be either niche-specific (the environment in *R. majalis*) or even common to other phylogenetically related bacterial species. Differences in gene expression indicate that exact genes bringing successful infection are quite different in these two closely related bacterial genera, and this is possibly due to the different genome sequences between the two bacterial species that are often resulted from the horizontal gene transfer or recombination.
Commonly expressed genes by *P. temperata* and *X. koppenhoeferi*. *tca* gene family encode four toxin complexes TcaA, TcaB, TcaC and TcaD (Bowen et al., 1998), but only *tcaC* was identified to be differentially expressed during grub infection in both bacteria. This supports the role of TcaC as either an activator or a chaperone in secretion of toxin complex from the cell (ffrench-Constant et al., 2003; Waterfield et al., 2001b). The hemolysin related proteins have been found to be important in the infection process either by regulating the bacterial survival or disrupting the blood cells (Fernandez et al., 2004; Rouault, 2004; Wilson et al., 2001). In *Photorhabdus luminescens* and *Xenorhabdus nematophila*, the major activity of hemolysin was revealed to be against insects’ hemocytes, but not as a major virulence factor (Brillard et al., 2001; 2002). Genes encoding hemolysin related proteins were identified to be up-regulated in *P. temperata* (*hemS*) and *X. koppenhoeferi* (*phlA*) suggesting the common roles played by these genes in the bacterial survival during infection. Surface-exposed components are known to be critical for establishment of disease in the host by causing resistance to immune defense (Rasmussen & Bjorck, 2001; Whatmore, 2001). Several outer membrane proteins (OMP) were up-regulated in both bacteria during interaction with grubs. OMPs were also identified to be up-regulated by *Moraxella osloensis*, a symbiont of the slug parasitic nematode *Phasmarhabditis hermaphrodita*, during infection to the slug *Deroberas reticulatum* (An et al., unpublished data). Flagellum-mediated motility is shown to provide a specific advantage for infection (Lee et al., 1998; Moens & Vanderleyden, 1996). Besides providing motility, flagella are important for bacterial attachment to
surfaces and are thus generally considered important virulence factors (Dalton and March, 1998, Moens and Vanderleyden, 1996). The isolation of genes fliI from P. temperata and fliM from X. koppenhoeferi indicates that the flagellar machinery is also important for both bacteria to establish interactions with the grub.

During infection, bacterial pathogens are frequently exposed to the host-imposed stress in the form of nutrient deprivation, oxidative stress, and immunological pressures (Buettner, 1993). Two survival factors, SurA and UspB were found to be differentially expressed in both bacteria. Gene surA encodes the enzyme peptidyl prolyl cis/trans isomerases (PPIase) (Ren et al., 2005). In nature, two classes of enzymes, the protein disulfide isomerases (Dsb) and PPIases, catalyze slow steps in protein folding in order to avoid accumulation of non-native protein conformations (Fischer et al., 1998; Schiene & Fischer, 2000). Studies from other bacteria have displayed importance of PPIase for bacterial survival during infection (Leuzzi et al., 2005; Ren et al., 2005). Capturing surA from both bacteria suggests its important role in promoting persistence of P. temperata and X. koppenhoeferi in the grub–imposed environment. Glutathione plays an important role in protection against oxidative stress and other stresses, such as detoxification of hazardous chemicals or heavy metals (Hayes et al., 2005; Hultberg, 1998; Marrs, 1996). Expression of genes related to glutathione production in this study suggests the possible mechanism for P. temperata and X. koppenhoeferi to protect them from stresses by detoxification during infection.

Genes, accA, aceE and thiA, identified in this study are linked to the generation of acetyl-coA which can be used as a carbon source (Ensign, 2006). Interestingly, gene
encoding the acetyl-coA synthesis protein was also found to be up-regulated in *M. osloensis* during infection to the slug (An et al., unpublished data). These data imply that acetyl-coA may play an important role in bacterial pathogenesis in invertebrates.

In this study, *dsbA* encoding a thiol-disulfide interchange protein and *dsbC* encoding a disulfide isomerase were identified from *P. temperata* and *X. koppenhoeferi*, respectively. DsbA is a strong thiol oxidant that catalyzes disulfide bond formation of proteins that are exported to the periplasm (Kadokura et al., 2003). DsbC participates in disulfide bond exchange which catalyses the folding of various factors including components of type II secreted subunits into effector proteins (Sandkvist, 2001). In *M. osloensis*, *dsbC* transcripts during slug infection were also identified (An et al., unpublished data). Actually, Dsb protein has been identified as a virulence factor in diverse bacterial pathogens associated with different order hosts (Alvarez *et al.*, 2006; Baltes & Gerlach, 2004; Stenson & Weiss, 2002; Yu & Kroll, 1999). Ubiquinone is a central component of the electron transport chain under aerobic conditions and functions in the formation of disulfide bonds in periplasmic proteins by facilitating the reoxidation of protein-disulfide isomerase (Alexander & Young, 1978; Sheehan *et al.*, 2003). Two genes, *nqrD* and *ubiM*, related to the ubiquinone synthesis pathway were identified to be up-regulated in *P. temperata* and *X. koppenhoeferi*, respectively, during grub infection. Considering that gene corresponding to the ubiquinone synthesis was also identified to be up-regulated in *M. osloensis* during infection to the slug (An et al., unpublished data), it is possible that genes encoding Dsb family and ubiquinone synthesis proteins are often co-expressed by bacterial pathogens during infection to invertebrate hosts.
The regulatory genes permit bacteria to respond to changes in their environment and are often associated with global regulatory systems as well as with regulation of virulence. The \textit{phoP} gene encodes the response regulator of the \textit{phoP/phoQ} two-component system which governs virulence by adapting to Mg$^{2+}$-limiting environment, and regulates numerous cellular activities in several Gram-negative species (Chamnongpol et al., 2003; Groisman, 2001). It was recently shown that a mutation in \textit{phoP} rendered \textit{P. luminescens} avirulent to insect larvae (Derzelle et al., 2004). Up-regulation of \textit{phoP} gene in both bacteria suggests its importance in regulation of bacterial virulence during grub infection.

Overview of the \textit{in vivo} gene expression by several other bacterial pathogens, including \textit{Actinobacillus pleuropneumoniae} in porcine lung tissue (Baltes & Gerlach, 2004), \textit{Moraxella osloensis} in the slug (An et al., unpublished data), \textit{Salmonella typhi} in human macrophages (Daigle \textit{et al.}, 2001), \textit{Escherichia coli} in the chicken (Dozois \textit{et al.}, 2003), and \textit{Mycobacterium} tuberculosis in human macrophages (Dubnau \textit{et al.}, 2002), indicated the common themes shared by \textit{P. temperata} and \textit{X. koppenhoeferi} during grub infection. Several genes are common not only to \textit{P. temperata} and \textit{X. koppenhoeferi}, but also to other phylogenetically related bacteria. For instance, outer membrane proteins are identified to be up-regulated in all bacterial species, and others include products of \textit{dsb} gene family in \textit{A. pleuropneumoniae} and \textit{M. osloensis}, two component systems in \textit{Salmonella}, and universal stress protein and acetyl-coA synthesis proteins in \textit{Mycobacterium}. In contrast, TcaC, SurA, ubiquinone synthesis proteins, glutathione, and hemolysin are more likely niche-specific genes up-regulated in the invertebrate host.
**Unique genes expressed by *P. temperata* or *X. koppenhoeferi***. Besides the above described common genes, there were many up-regulated genes that were different between *P. temperata* and *X. koppenhoeferi* during infection to *R. majalis*. Although *X. koppenhoeferi* grew slower than *P. temperata*, both bacteria had similar virulence against *R. majalis*. Possibly, this could be in part due to the different genes up-regulated by these two bacteria during infection of *R. majalis*.

Bacterial phospholipase is a key virulence factor in bacteria-induced haemolysis, and this activity likely provides a favorable source of nutrients for bacterial growth in addition to providing a suitable environment for replication and survival (Istivan & Coloe, 2006). The differentially expressed gene *pplA* encoding phospholipase A in *P. temperata* may contribute to its faster growth rate compared to *X. koppenhoeferi* during infection to *R. majalis*.

The isolation of genes related to iron uptake reflects the importance of iron for bacterial growth in the grub. ABC transporters play a major role in iron acquisition pathways – a common theme in bacterial pathogenesis is the up-regulation of genes involved in this function. Besides involvement in uptake of nutrients, ABC transporters have also been implicated in the infection process of some pathogens by facilitating adhesion to host cells (Jenkinson, 1992; Marra *et al.*, 2002; Pei & Blaser, 1993). Thus, expression of these genes in the grub may be important in obtaining iron for bacterial survival and regulation. However, although genes involved in iron uptake were found to be induced in both bacteria, genes encoding the siderophore-dependent proteins were
only differentially expressed in *P. temperata*. The siderophore is usually produced
during infection in order to scavenge iron that is bound to transferrin in the haemolymph
(Nichol et al., 2002), and this may suggest that the iron is more limited to *P. temperata*
than to *X. koppenhoeferi* during infection to *R. majalis* due to their different growth rates.
In addition, gene *resP* identified in *X. koppenhoeferi* is similar to genes encoding putative
resistant proteins, and the pathway network displayed its role in iron transportation.
Unlike siderophore dependent factors, expression of *resP* in the grub may be an
alternative means to help *X. koppenhoeferi* obtain iron in the haemolymph.

Many bacterial pathogens use type III secretion machinery to deliver virulence
effector proteins across the bacterial cell envelope into host cells (Alfano & Collmer,
2004; Buttner & Bonas, 2003; Collmer et al., 2002; Hueck, 1998). The gene *ttsL*
encoding components of type III secretion machineries was only identified from *P.*
*temperata* in this study. Further, several genes encoding proteins with putative functions
of virulence or secretion, e.g. *toxC, lspA* and *virB* from *P. temperata* and *virH* and *
rtxC* from *X. koppenhoeferi* were also identified in this study. The pathway network analysis
indicates that they are related to the bacterial virulence. The differences in expression of
virulence and secretion related genes imply the differential infection mechanisms
between the two bacteria.

Except *surA, gluS (gluR)* and *uspB* which are common to both bacteria, a few
genes encoding heat shock proteins and other stress proteins were identified to be
differentially up-regulated in either *P. temperata* or *X. koppenhoeferi* only. Gene *
sspA* encoding the protein responsible for starvation and *hspC* encoding a heat shock protein
were identified in *X. koppenhoeferi* and *P. temperata*, respectively. Up-regulation of gene *trx*B encoding thioredoxin reductase suggests that *X. koppenhoeferi* experienced increased oxidative stress during the grub infection. Thioredoxin reductase constitutes a thiol-dependent oxidation reduction system that catalyzes the reduction of certain proteins (Holmgren, 1985), and reduces disulfide bonds that form spontaneously in aerobically grown cultures (Prinz et al., 1997). For *P. temperata*, up-regulation of *dsbA* gene may also respond to the increased oxidative stress during the grub infection. Gene *msrA* encoding the peptide methionine sulfoxide reductase was only identified in *X. koppenhoeferi*. The peptide methionine sulfoxide reductase is an important antioxidant enzyme that mediates the repair of proteins damaged by sulfoxidation of methionine residues (Alamuri & Maier, 2004; Weissbach *et al.*, 2002). The pathway network analysis showed that besides regulating the bacterial survival and virulence, *msrA* has connectivity with other genes such as *rpoB* which was also identified to be differentially expressed in *X. koppenhoeferi* during infection. *clpP* encodes an ATP-dependent serine protease which is normally involved in the degradation of damaged polypeptides and the salvage of amino acids (Gottesman & Maurizi, 1992), and it was also found to be up-regulated in response to heat shock or ethanol stress (Gerth et al., 1998). Increased expression of *clpP* and *msrA* may reflect the production of damaged proteins arising during *X. koppenhoeferi* infection to grubs. In *P. temperata*, response to the oxidative damage may be fulfilled by the differentially expressed gene *topA* encoding DNA topoisomerase I which contributes to DNA supercoiling, global gene transcription, bacterial survival against high osmolarity and oxidative damage (Bhriain & Dorman,
Differences in up-regulation of these genes imply variations in response between the two bacteria upon infection to the same grub host *R. majalis*.

LPSs are well-defined virulence factors playing a major role in the development of septicemia (Mayeux, 1997). The bacteria infected insects die mainly due to a septicaemia dependent on the bacterial growth, and sometimes a bacterial toxaemia precedes the resulting septicaemia (Forst, 1997). In *M. osloensis*, Tan and Grewal (2002a; 2002b) reported that the purified LPS was toxic to the slug with an estimated 50% lethal dose of 48 μg when injected into the shell cavity. In a study with *Xenorhabdus nematophila*, LPS was toxic to the hemocytes of *G. mellonella* larvae only after a critical level was released (Dowds & Peters, 2002; Dunphy & Webster, 1988; Halwani & Dunphy, 1997). Thus, LPS may act in concert with other factors to destroy hemocytes (Dowds & Peters, 2002; Ribeiro et al., 1999), which can also be supported by the pathway network analysis that *lpsE* plays roles in virulence via regulating other genes.

Increased gene expression involving lipopolysaccharide (LPS) biosynthesis was found only in *X. koppenhoeferi* during infection to the grub. This may explain in part that relatively slower growing *X. koppenhoeferi* did not differ from *P. temperata* in virulence to *R. majalis* because the increased expression of *lpsE* may complement the growth dependent septicaemia.

While two regulatory genes *cpxR* and *phoP* were identified in *P. temperata*, only *phoP* was identified in *X. koppenhoeferi*. CpxR is one part of two-component CpxA/CpxR system found in many Gram-negative bacterial pathogens, and this two-component system senses cell envelope stress and up-regulates a number of periplasmic
factors to maintain, adapt and protect the bacterial envelope in response to a variety of stressors (Raivio, 2005). Identification of cpxR only in P. temperata again indicates the different stress response mechanisms between the bacterial species when facing the same host imposed environment.

In summary, although differences in gene expression between closely related bacteria P. temperata and X. koppenhoeferi were observed in this study, these unique genes were found to be up-regulated in other unrelated bacteria. For example, topoisomerase, type III secretion systems, and LPS synthesis proteins were found to be up-regulated in many bacterial pathogens, but they are unique to either P. temperata or X. koppenhoeferi. This interesting phenomenon could be possibly explained by the horizontal gene transfer.

Pathway network analysis. As the exact nature of host-pathogen interactions in an invertebrate host is not well established, the pathway networks build in this study were mainly based on the extensive literatures on the genes expressed by pathogenic bacteria in higher order hosts. Thus, by tracking the references used in the pathway analysis, the interesting common themes in bacterial pathogenesis across a variety of host-pathogen interfaces could be demonstrated. Based on the pathway analysis, the common themes for virulence and secretion include outer membrane structures and toxin complexes, ABC transporter for iron acquisition, survival proteins and DNA topoisomerase for stress response, two component systems for regulation, products of dsb gene family for protein confirmations, and acetyl-CoA synthesis proteins for nutrient metabolisms.
6.6 FUTURE DIRECTION

A total of 65 genes, including common genes, were identified to be up-regulated in *P. temperata* and *X. koppenhoeferi* during infection of *R. majalis*. The pathway analysis would provide valuable directions for future research by establishing a functional basis for the expressed genes in certain pathways. Although most of the identified genes in this study are functionally connected in the pathway network, suggesting their relevance in the same biological process, some genes are more likely involved in regulation of virulence while others may be involved in global regulation of general cell processes during infection.

For convenience, we termed nexus genes as the genes to regulate several other genes in the pathway achieving successful infection. As nexus genes often function as pivotal nodes in a complex network, altering the function of a nexus gene by knockout will have more profound consequences than a less connected gene (King et al., 2005). Our analysis suggests several nexus genes in the pathway that would be candidates for further analysis, and these nexus genes include genes *abcA* and *aceE* from *P. temperata* and genes *msrA*, *lpsE*, and *rpoB* from *X. koppenhoeferi*.

In addition, as several up-regulated genes, including *gluS*, *dsbA*, *thiA*, *topA*, *cpxR*, and *phoP* from *P. temperata* and *sspA*, *phoP*, *dsbC*, and *accA* from *X. koppenhoeferi*, potentially regulate multiple cell processes in the pathway network but are not identified to be nexus genes in current database, we hypothesize that these would be necessary genes involved in the infection. Thus, deletion in one or several of these genes could
weaken the bacterial virulence, and these would be potential candidates for further analysis.

However, we should keep in mind that the major drawback of the network analysis is the reliance on published literature, indicating that the genes with more connectivity in the network are not because these genes are more fundamental or biologically important but because they have been studied more. Therefore, the candidate genes indicated by pathway analysis would be studied first in detail in the future to make a backbone for the network, and then the potential candidate genes could be studied to build a clear pathway, and finally the little-studied genes including resP, uspB, gluR, ubiM, and lysC identified in this study may also emerge as potential candidates for analysis in the future.

6.7 REFERENCES


Figure 6.1: Functional associations among genes up-regulated in *P. temperata* GPS11 during infection to *R. majalis*. The gene expression data identified by SCOTS analysis were analyzed by PathwayStudio software using bacteria database. Graphical output delineates a functionally related network of genes. Gene IDs followed by a capital P meaning *P. temperata* (according to Table 6.1) are comparative to Entrez Gene IDs and presented as object nodes, and function relations are presented as lines. Red ellipse symbols indicate proteins, yellow rectangles represent cell processes, orange hexagons represent functional class, pink o-vertex means regulator, purple dots indicate binding, grey rectangles indicate regulation, and blue rectangles indicate expression.
Figure 6.2: Functional associations among genes up-regulated in *X. koppenhoeferi* AMK11 during infection to *R. majalis*. The gene expression data identified by SCOTS analysis were analyzed by PathwayStudio software using bacteria database. Graphical output delineates a functionally related network of genes. Gene IDs followed by a capital X meaning *X. koppenhoeferi* (according to Table 6.2) are comparative to Entrez Gene IDs and presented as object nodes, and function relations are presented as lines. Red ellipse symbols indicate proteins, yellow rectangles represent cell processes, orange hexagons represent functional class, pink o-vertex means regulator, purple dots indicate binding, grey rectangles indicate regulation, and blue rectangles indicate expression.
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