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PHOTORHABDUS LUMINESCENS PUTATIVE *MAD* FIMBRIAE ARE REQUIRED FOR ADHERENCE IN THE MATERNAL INTESTINE AND INITIATION OF SYMBIONT TRANSMISSION IN *HETERORHABDITIS BACTERIOPHORA*

presented by

Bettina Maria Kaufmann-Daszczuk

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Major Professor's Signature

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Bettina Maria Kaufmann-Daszczuk

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ABSTRACT

PHOTORHABDUS LUMINESCENS PUTATIVE *MAD* FIMBRIAE ARE REQUIRED FOR ADHERENCE IN THE MATERNAL INTESTINE AND INITIATION OF SYMBIONT TRANSMISSION IN *HETERORHABDITIS BACTERIOPHORA*

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The nematode *Heterorhabditis bacteriophora* lives in a symbiotic relationship with the entomopathogenic bacterium *Photorhabdus luminescens*, which colonizes and persists in the intestine of *H. bacteriophora* infective juveniles. To identify the genes involved in colonization of *H. bacteriophora*, ~8000 *Photorhabdus* spp. mutants were constructed by transposon mutagenesis and screened for symbiont transmission. Twelve of the 30 **tr**a**n**smission mutants (TRN) contained transposon insertions in the putative fimbrial gene cluster VI, comprised of genes *madR* through *madJ*. While *P. luminescens* has eleven fimbrial gene clusters, only gene cluster VI encoded fimbriae might be essential for symbiont transmission. When one of the TRN mutants was complemented with *mad* genes, it was able to adhere to the maternal *H. bacteriophora* intestine once again, but no transmission of the complemented mutant from mother to offspring was observed. All TRN mutants displayed characteristics of wild-type *P. luminescens* such as bioluminescence, pigmentation, and colony morphology. All TRN fimbrial mutants were able to form a biofilm on an abiotic surface and were shown to hemaglutinate blood cells. In addition, an inverted repeat switch, found just upstream of *madA*, controls gene cluster VI during phase variation (ON ↔ OFF switching of phenotype expression). Overall, the results of this study suggest that fimbriae encoded by gene cluster VI are nematode specific and important during colonization.

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I would like to dedicate this thesis to my husband Joseph Daszczuk and my mother Maria Kaufmann, the two most important people in my life.

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CHAPTER 1: INTRODUCTION

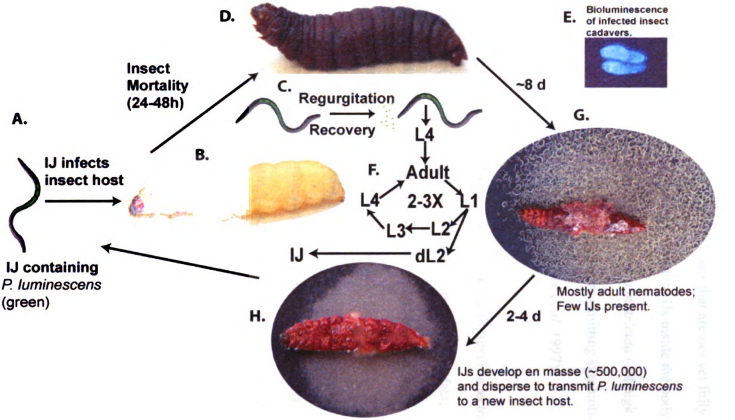
The introduction is divided into six parts. Part I describes the symbiotic relationship of *Photorhabdus* spp. and *Heterorhabditis bacteriophora*. Part II explains how symbionts are transmitted during different stages of *H. bacteriophora* development. Part III introduces the importance of phenotypic variation in *P. luminescens*. Part IV summarizes the general role of fimbriae in transmission. Part V describes the major types of pili and fimbriae and biosynthesis pathways. Part VI explains why the nematode-bacteria symbiosis is an excellent model to study the role of fimbriae in human pathogens.

INTRODUCTION

Photorhabdus spp. *and* Heterorhabditis bacteriophora *symbiosis*

The Gram-negative bacteria *Photorhabdus luminescens* and *Photorhabdus temperata*, of the family Enterobacteriaceae, are symbiotically associated with the nematode *Heterorhabditis bacteriophora* (Khan & Brooks, 1976; Poinar, 1975). In this obligate mutualism, the nematodes require the bacteria for growth, reproduction, and insect killing, while the bacteria require the nematode host as a vector for transmission to an insect prey (Han and Ehlers, 2000; Ciche *et al.* 2001). Together, *P. luminescens* and *H. bacteriophora* are able to infect and kill a large variety of insect larvae (Khan and Brooks, 1976; Poinar, 1975). In soil, *H. bacteriophora* persists as the developmentally arrested infective juvenile (IJ) stage, which transmits *P. luminescens* cells in its intestine to insect hosts (Figure 1-1; Hatab *et al.* 1998). When IJs encounter insect hosts, they migrate into the insect's hemocoel either by means of natural openings, such as the mouth or anus, or by penetrating the outer cuticle (Abu Hatab *et al.* 1995). Once inside the larval body, the IJs regurgitate their symbiotic bacteria into the hemolymph of the insect, where they infect and kill the insect within 24-48 hours (Figure 1-1; Johnigk and Ehlers, 1999; Ciche and Ensign, 2003). While the bacteria actively divide in the insect's hemolymph, they produce and secrete a large variety of toxins, antibiotics, antifungal compounds, nematicidal compounds, and enzymes, which function in the protection, preservation, and degradation of the insect cadaver (Forst and Nealson, 1996; Richardson *et al.* 1988). Within this nutritionally favorable and protective environment, the developmentally arrested IJs exit from diapause and resume development into egg-laying hermaphroditic adults (Johnigk and Ehlers, 1999). Eggs deposited into the highly

Figure 1-1: Lifecycle of *H. bacteriophora* (from Ciche). In the soil, infective juveniles (IJs) carry *P. luminescens* in their intestine (A). When IJs encounter an insect host, they regurtitate *P. luminescens* into the hemolymph, thereby killing the insect (C). Environmental cues, not fully understood yet, trigger the recovery process, and IJs (developmentally arrested in stage L2) go through stages L3 and L4 and finally turn into egg-laying adult hermaphrodites (F). Laid eggs hatch and complete stages L1 through L4 and develop into males and egg-laying females. After several generations, eggs are no longer laid into the environment, but instead develop within the body of the mother by a process called *endotokia matricida*. The resulting IJs emerge from the insect cadaver in search for a new insect host (H).



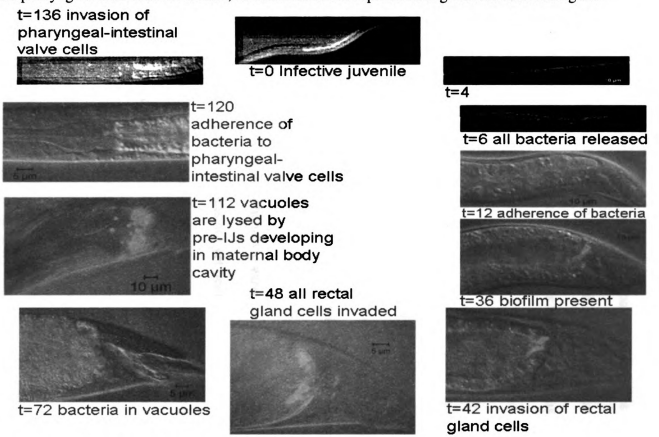
nutritious insect cadaver develop into males, females, or hermaphrodites (Johnigk and Ehlers, 1999).

After several generations, in response to environmental cues that are not yet fully understood, eggs are no longer laid but instead hatch and develop into IJs inside the body of adult hermaphrodites and females – a process known as *endotokia matricida* (Johnigk and Ehlers, 1999). The resulting IJs leave the insect cadaver, most transmitting symbiotic bacteria and actively hunt for a new insect host (Figure 1-1; Boemare *et al.* 1997).

Symbiont transmission

Entomopathogenic nematodes, such as *H. bacteriophora*, are highly specific as to which bacteria they associate with (Akhurst, 1983; Gerritsen and Smits, 1997). In fact, when the nematodes are given a mixture of symbiotic and non-symbiotic bacteria, only the symbiotic bacteria are transmitted to the IJs (Ciche *et al.* 2006). In the IJ stage of nematode development (Figure 1-2; Ciche *et al.* 2006), symbiotic bacteria are located in the anterior intestine immediately posterior to the pharyngeal bulb (Johnigk and Ehlers, 1999), but are also distributed throughout the intestines. After recovery, during which the nematode regurgitates all of the symbiotic bacteria, *H. bacteriophora* are recolonized by *P. luminescens* at the most posterior end of the intestine, near the rectal gland cells, by adherence of symbiotic bacteria (Figure 1-2; Ciche *et al.* unpublished data). The symbionts actively reproduce in the posterior intestine of *H. bacteriophora* until about 42 hours post IJ recovery, when the biofilm-like aggregate of *P. luminescens* begins to invade the rectal gland cells of the adult nematode (Figure 1-2). The bacteria continue to divide and persist inside vacuoles within the rectal gland cells.

Figure 1-2: Location of GFP-labeled *P. luminescens* bacteria within *H. bacteriophora* during development (adapted from Ciche). Time (t) is in hours. In the infective juvenile, bacteria are distributed throughout the intestine. After release of symbionts within the insect host, *P. luminescens* recolonizes the posterior end of *H. bacteriophora* intestine by attachment of a single bacterium. The bacteria actively reproduce and form a biofilm-like structure near the rectal gland cells. This biofilm can invade *H. bacteriophora* rectal gland cells, where *P. luminescens* establishes a reservoir of itself within vacuoles. During development of pre-IJ's within the mothers body, vacuoles containing *P. luminescens* are lysed and the bacteria adhere to the pharyngeal-intestinal valve cells of IJ's. After invasion of the pharyngeal-intestinal valve cells, *P. luminescens* can spread throughout the intestine again.



Pre-IJs, which develop from eggs inside the mother's body cavity, gain access to symbiotic bacteria when vacuoles containing *P. luminescens* lyse. While still in the mother's body, pre-IJs acquire *P. luminescens* by feeding on the body contents that are mixed with bacteria. During this feeding, a single *P. luminescens* bacterium adheres to and eventually invades the pharyngeal-intestinal valve cells (Figure 1-2). From there, *P. luminescens* can recolonize and persist in the intestinal lumen of IJs until the next insect host is found.

Phenotypic variation in P. luminescens

In order to adapt to changing environments, a variety of bacteria undergo phase variation, which is usually a high-frequency ON ↔ OFF switching of phenotype expression in most bacterial species (Henderson *et al.* 1999). Phase variation is a random event and results in a mixed bacterial population of phenotypes, allowing the population to quickly adapt to more than one environment (Henderson *et al.* 1999). In *P. luminescens*, the switch from primary to secondary phenotyic variants occurs at low frequency, and reversal from secondary to primary has not yet been reported (Joyce *et al.* 2005). Only primary phenotypic variants are transmitted to *H. bacteriophora* IJs and support nematode reproduction and development (Han and Ehlers, 2001). Secondary phenotypic variants also differ from the primary variants by repression of many other primary phenotypes such as crystalline inclusion proteins, antibiotic production, pigmentation, and bioluminescence (Akhurst, 1983). It is important to be able to distinguish between the two variants when working with *P. luminescens*. If phenotypic variants are not carefully selected for experiments, the resulting data can be misleading or inconclusive.

Phase variation in bacteria in general can occur by several mechanisms by which gene expression is regulated. One mechanism is known as slipped-strand mispairing, which employs a series of repetitive DNA segments for translational control resulting in the alteration of the reading frame and protein expression (Henderson *et al.* 1999). For example, promoter strength of *nadA* in *Neisseria meningitides* strain MC58 is reduced by a change in the unit repeat number upstream of the promoter region (Martin *et al.* 2003). Another mechanism is the differential methylation of GATC sites by deoxyadenosine methylase. Depending on whether or not GATC sites are methylated, genes are either expressed or not (van der Woude *et al.* 1996). For example, expression of P fimbriae in *E. coli* is regulated by differential methylation depending on temperature. At 37°C, P fimbriae are expressed, but no expression takes places at temperatures below 26°C (Blyn *et al.* 1990; van der Woude *et al.* 1996). Environmental signals can also affect the switch frequency of phase variation. For example, low iron concentrations initiate phase variation in *N. gonorrhoeae* pili during the infection process, allowing this human pathogen to rapidly adapt to environmental conditions within a host (reviewed in van der Woude and Bäumler, 2004).

While random phase variation affects more than one phenotypic characteristic, antigenic variation, a process related to phase variation, results in the differentiated expression of surface organelles (Henderson *et al.* 1999). One of the earliest examples of antigenic variation of flagellar biosynthesis was found in *Salmonella* spp. that exhibited diphasic agglutination stages due to variation of flagellar biosynthesis (Zieg *et al.* 1977). Antigenic variation of type 1 fimbriae in *E. coli* is accomplished by inverting the promoter region upstream of *fimA*, thereby turning the operon ON or OFF (Abraham *et*

al. 1985). Expression of *P. luminescens* fimbrial gene cluster VI, which has a recombinase homologous to FimB found in the *fim* operon in *Escherichia coli* (based on a *coli*BASE search), could also be regulated by such an invertible switch. Antigenic variation of the *mad* fimbriae in *P. luminescens* might enable a subset of the bacterial population to express a fimbrial adhesin allowing them to colonize a specific receptor expressed in the *H. bacteriophora* posterior intestine.

General role of fimbriae in transmission

Bacterial attachment to a eukaryotic host cell is the first step in the colonization process of many bacteria (Yamashiro and Iwanaga, 1996). Attachment by means of pili or fimbriae is a common mechanism found in various Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *E. coli*, but can also be utilized by some Gram-positive bacteria, such as *Enterococcus faecalis* (Handley and Jakobs, 1981; Woods *et al.* 1980). The nematode symbiont *P. luminescens* has eleven gene clusters predicted to encode proteins related to known pili and fimbriae biosynthesis (Figure 1-3; Duchaud *et al.* 2003). Gene cluster IV consists of putative pseudogenes and is probably not functional (Figure 1-3). Gene clusters II, III, VII, and VIII encode fimbrial like proteins of unknown function (Figure 1-3), but the presence of an usher and chaperone suggest that these putative fimbriae are assembled via an usher-chaperone type pathway. Cluster I and V encode proteins similar to mannose-resistant/Proteus-like (MR/P) fimbriae, also predicted to be assembled by an usher-chaperone pathway (Figure 1-3). In humans, *Proteus mirabilis* is a common cause of urinary tract infection, and the bacteria express MR/P fimbriae especially during the infection process (reviewed by Zhao *et al.* 1997). Cluster VI, characterized in this study, is also likely to be assembled by an usher-chaperone

pathway. Cluster IX fimbriae are similar to Type I fimbriae, commonly found in uropathogenic bacteria (Figure 1-3). The pili encoded by clusters X and XI are similar to Type 4 pili that enable twitching motility in *Ps. aeruginosa*, a common human pathogen (Figure 1-3).

This large number of gene clusters devoted to the biosynthesis of pili and fimbriae is uncommon in bacteria. For example, *Salmonella enterica* serovar Typhimurium has five fimbrial gene clusters, and the uropathogenic *P. mirabilis* produces four types of fimbriae (Ledboer *et al.* 2006; Massad *et al.* 1996). On the other hand, *E. coli* O157:H7 was recently found to have a total of 16 fimbrial gene clusters (Low *et al.* 2006). Perhaps bacteria benefit from having multiple gene clusters encoding pili and fimbriae, for it allows them to attach to different types of surfaces and tissues. For example, variation in surface appendages in *Neisseria* spp. modulates tissue tropism and allows the bacteria to adapt to different environments (reviewed in Dehio *et al.* 2000). Also, symbiotic bacteria might require specialized pili to switch from being a symbiont to being a pathogen. For example, phase variation of surface components in *N. meningitides* results in a pathogenic phenotype (de Vries *et al.* 1996). While the exact role of fimbriae in the establishment of symbiosis is currently not clear, fimbriae are expected to play a key role in the establishment of symbiotic host-bacterial interactions.

Figure 1-3: *Photorhabdus luminescens* fimbrial gene clusters (adapted from Duchaud *et al.* 2003). Gene functions are given at the top. Genes of similar functions are colored the same. Cluster VI was renamed from *plu* to *mad* (**m**aternal **a**dhesion **d**eficient).

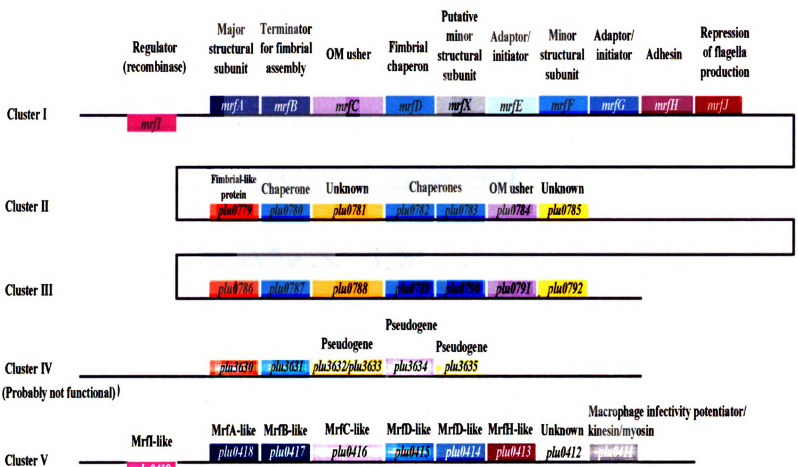


Figure 1-3: continued

Major types of pili and fimbriae and biosynthesis pathways

While the variety of pili and fimbriae is large and new types of adhesive structures are discovered continuously, the best-characterized types are type I fimbriae, P pili, type IV pili, and curli. Type I fimbriae function in invasion and persistence of uropathogenic bacteria in host cells (Baorto *et al.* 1997; Martinez *et al.* 2000). These fimbriae are distributed all over the bacterial surface and vary in length from 0.2 to 1.0 μm (Sharon, 1987). The rod of type I fimbriae consists of 500 to 3000 copies of the major structural subunit, FimA, that are arranged in a right-handed helix about 6.9 nm in diameter (Capitani, 2006). The linear tip fibrillum consists of several copies of FimG and FimF, topped by the adhesin protein FimH (Jones *et al.* 1995). The chaperone FimC binds fimbrial subunits and prevents degradation and premature folding (Jones *et al.* 1993). FimC delivers each subunit to the outer membrane usher FimD where it is channeled to the bacterial surface and incorporated in the growing fimbriae (Jones *et al.* 1993).

Alongside Type I fimbriae, uropathogenic bacteria also express P pili. P pili are flexible pili that can elongate when exposed to mechanical stress and spontaneously contract when stress is absent (Jass *et al.* 2004; Fällman *et al.* 2005). This ability of P pili enables *E. coli* to withstand the rinsing action of urine in the urinary tract and allows them to colonize epithelial host cells (Fällman *et al.* 2005). P pili are assembled on the bacterial surface into right-handed helices of repeated PapA subunits with a tip consisting of mostly PapE subunits (reviewed in Soto and Hultgren, 1999). Assembly of P pili is achieved via the general secretory pathway. During this process, pilin subunits are secreted across the inner cell membrane, where they bind to a chaperone and are taken to

the usher (Hultgren *et al.* 1991). The resulting pilin fiber consists of ~10^3 PapA subunits organized into a right-handed helix (reviewed in Fällman *et al.* 2005). The tip fibrillum consists of subunits PapK, PapE, and PapF, topped with the adhesion subunit PapG (Hultgren *et al.* 1991). Both type I fimbriae and P pili act as virulence factors and are commonly found in uropathogenic *E. coli* and other members of the *Enterobacteriaceae* family (Kallenius *et al.* 1981; Krogfelt *et al.* 1990).

Type IV pili are utilized by a number of human pathogens, such as *Pseudomonas aeruginosa*, and are involved in activities such as eukaryotic cell colonization and phage adsorption, but their most notable function is in twitching motility (Smyth *et al.* 1996). When pathogens lose Type IV pili or their ability to move by twitching motility they become avirulent (reviewed in Mattick *et al.* 1996). Type IV pili are located at the pole of bacterial cells, consist of a single repeated pilin subunit PilA arranged in a helical conformation, and vary in length from ~1.0-4.0 μm (Mattick *et al.* 1996). They are assembled via the general secretion pathway (Soto and Hultgren, 1999). The prepilin peptidase PilD cleaves a short leader peptide from the subunits (Alm and Mattick, 1997). The inner membrane protein PilC binds the cleaved pilin subunit and serves as a platform for assembly (Alm and Mattick, 1997). PilB, a nucleotide-binding protein, facilitates secretion of the pilus through the outer membrane channel PilQ (Alm and Mattick, 1997). While all Type IV pili have similar quaternary and tertiary structure, they exhibit some primary and secondary structural differences depending on species and strains (Mattick, 2002).

Curli, a class of thin and irregular pili, are found on the surface of a variety of clinical isolates of human pathogens, such as *E. coli* and *Salmonella enteritidis*, that

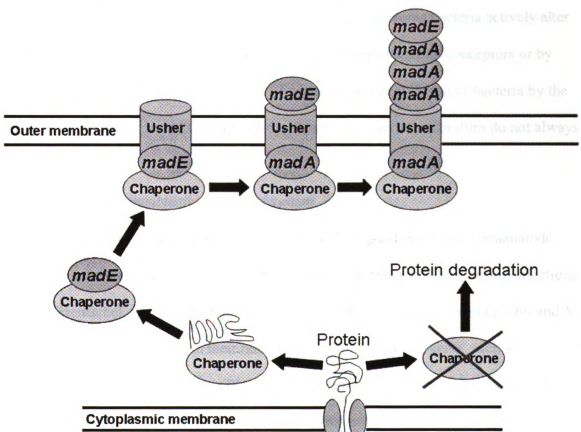
allow them to bind to a large variety of host proteins (reviewed in Soto and Hultgren, 1999). Curli pili are assembled by the extracellular nucleation-precipitation pathway (Soto and Hultgren, 1999). Individual subunits of CsgA are secreted into the extracellular environment where they are nucleated by CsgB to form a fiber (reviewed in Barnhart and Chapman, 2006). Accessory proteins CsgE, CsgF, and CsgG function during secretion of CsgA and assembly of the curli fiber, but their exact roles are not yet known (Barnhart and Chapman, 2006). Regulation of curli expression is dependent on a variety of environmental cues and is under the control of the transcriptional regulator CsgD (Hammar *et al.* 1995). Curli can bind to many different host proteins and are proposed to play a key role in invasion of host cells (Bian *et al.* 2000).

Photorhabdus luminescens mad genes are homologous to *fim* genes in *E. coli*, and the *mad* gene cluster contains genes predicted to encode an usher and three chaperones. The presence of the usher and the chaperones lead to the conclusion that *mad* fimbriae are likely to be assembled by an usher-chaperone pathway. A hypothetical biosynthesis pathway for fimbriae encoded by *P. luminescens* gene cluster VI is given in Figure 1-4. Pre-fimbrial Mad protein is secreted into the cytoplasm, where it binds to its chaperone (Figure 1-4). The chaperone prevents protein degradation and promotes proper folding of pre-fimbrial proteins into *mad* subunits (Figure 1-4). The chaperone transports *mad* subunits to the outer membrane usher, which channels it to the outside of the bacteria where the subunits assemble into *mad* fimbriae (Figure 1-4).

While pili and fimbriae continue to be the most common form of bacterial adhesins, several nonfimbrial adhesins, such as filamentous hemagglutinin, mannose-

resistant hemagglutinin, and invasion proteins have been discovered (reviewed in Finlay and Falkow, 1989).

Figure 1-4: Putative biosynthesis pathway of *mad* fimbriae via the usher-chaperone pathway. Individual subunit proteins are secreted into the periplasm, where they bind to a chaperone. The chaperone prevents degradation, promotes proper folding, and transports the subunit to the outer membrane usher. The fimbrial subunits migrate through the usher to the bacterial surface, where they assemble into the final structure.



Once bacteria are attached to a host cell surface, they can persist, often as a biofilm, and sometimes invade the host cells. The term biofilm can be broadly applied to any community of microorganisms that can attach to a range of biotic and abiotic surfaces (O'Toole *et al.* 2000). Within the biofilm, bacteria are often encased by an extracellular matrix, often consisting of polysaccharides and proteins, and assemble into

highly complex structures (O'Toole *et al.* 2000). Induced by surface attachment, bacteria undergo changes in gene expression, such as down regulation of flagella synthesis, increased exopolysaccharide synthesis, and antibiotic resistance (reviewed by O'Toole *et al.* 2000). In the nematode intestine, in addition to many other benefits, the biofilm protects *P. luminescens* from the shear forces that occur during frequent defecation. Invasion can take place by many mechanisms. For example, some bacteria actively alter the plasma membrane of the host cell by either contacting cell surface receptors or by injecting a protein into the host cell, both of which trigger engulfment of bacteria by the host cell (Pizarro-Cerdá and Cossart, 2006). However, bacteria in a biofilm do not always invade host cells.

Nematode-bacteria symbiosis as a model system

Some of the same processes that take place during colonization of a nematode host by its symbiotic bacteria are the same processes used by pathogens during infectious disease (Hentschel *et al.* 2000; Bennett and Clarke, 2005). *Neisseria meningitides* and *N. gonorrhoeae* cause infection in their human host by a cascade of events, such as adhesion, invasion, and signaling (Plant and Johnsson, 2003). In the same manner, *P. luminescens* adheres to rectal gland cells in *H. bacteriophora* and forms a biofilm that ultimately invades the rectal gland cells. Uropathogenic *E. coli* cause urinary tract infections in humans using Type I pili to attach to and invade epithelial cells in the urinary tract, where they establish a reservoir of *E. coli* cells that can cause recurrent infections in the human host (Mulvey *et al.* 1998). In a similar manner, *P. luminescens* persist in vacuoles within invaded rectal gland cells (Figure 1-2).

Pseudomonas aeruginosa, an opportunistic human pathogen, attaches to the

surface of mucosal host cells using Type IV pili and is involved in infections most frequently associated with burn victims, cancer patients, cystic fibrosis, and other immune-compromised individuals (Bodey *et al.* 1983; Pier, 1985; Irvin, 1993; Paranchych *et al.* 1986; Lee *et al.* 1989). *Burkholderia cenocepacia* uses cable pili to attach to the lung epithelial cells of cystic fibrosis patients (Clode *et al.* 2000; Sun *et al.* 1995). Based on these similarities to human pathogens, the nematode-bacteria-insect system could serve as a model system for the role of fimbriae in the pathogenicity of human pathogens.

This model system has other advantages as well. The entire genome of *P. luminescens* TT01 has been completely sequenced and is publicly available, simplifying molecular and genetic work tremendously. Also, *H. bacteriophora* is transparent and allows for detection of even single GFP-labeled *P. luminescens* throughout the entire body of the nematode. In the lab, *H. bacteriophora* and *P. luminescens* can be cultured separately, and *H. bacteriophora* is an excellent model organism for it has a short development time and is easily maintained.

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CHAPTER 2: *PHOTORHABDUS LUMINESCENS* PUTATIVE *MAD* FIMBRIAE ARE REQUIRED FOR ADHERENCE AND TRANSMISSION IN *HETERORHABDITIS BACTERIOPHORA*

This chapter is formatted to be published in the *Journal of Bacteriology*. This study reported for the first time that a putative type of fimbriae, *mad* fimbriae, is required for host colonization and symbiont transmission. *Photorhabdus luminescens* mutants with a defect in *mad* genes cannot adhere to the posterior maternal intestine and consequentially are not transmitted to infective juveniles that develop inside the maternal body cavity. This inability to adhere in *H. bacteriophora* ultimately hinders symbiont transmission. When a *P. luminescens* mutant was complemented with *mad* genes, adherence to the maternal intestine was partially restored. In addition, this study is the first to report the involvement of an inverted repeat switch in the regulation of the *mad* gene cluster during phase variation. The presence of this switch was made obvious by the presence of a recombinase included in the *mad* gene cluster. In primary *P. luminescens* phenotypic variants, the inverted repeat was in the ON position and OFF in secondary phenotypic variants.

Title. *Photorhabdus luminescens* putative *mad* fimbriae are required for adherence and transmission in *Heterorhabditis bacteriophora*

BETTINA M. KAUFMANN-DASZCZUK[1], KWI S. KIM[1], AND TODD A. CICHE[1]*

[1]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824

*Corresponding author: Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, Phone: (517) 355-6463 x1569, Fax: (517) 353-8957, E-mail: ciche@msu.edu.

ABSTRACT

The nematode *Heterorhabditis bacteriophora* lives in a symbiotic relationship with the entomopathogenic bacterium *Photorhabdus luminescens*. In this obligate mutualism, *P. luminescens* colonizes and persists in the intestine of *H. bacteriophora* infective juveniles by transmission of bacterial symbionts from mother to offspring. In order to identify symbiont genes required for transmission, ~8000 transposon mutants (labeled with GFP) were screened for a decreased efficiency in transmission to *H. bacteriophora* infective juveniles (IJs). Thirty **tra**nsmission mutants (TRN) were obtained defective in transmission to IJs. Twelve of the TRN mutants contained transposon insertions in one of ten putative fimbrial gene clusters, VI, comprised of genes *madR* through *madJ* (**m**aternal **a**dhesion **d**eficient) and predicted to encode an usher-chaperone assembled fimbriae. All but two of these twelve mutants were totally defective in transmission and were unable to adhere to the maternal intestine to initiate transmission. One of the TRN fimbrial mutants, TRN26-25 (*madJ* defective), was able to adhere to the maternal intestine again when it was complemented with wild-type *mad* genes. An inverted repeat promoter switch located upstream of *madA* was determined to be ON in *P. luminescens* primary phenotypic variants and OFF in secondary phenotypic variants. RT-PCR of *P. luminescens* primary phenotypic variants revealed expression of *mad* genes in wild-type and TRN26-25 bacteria. Despite having a mutation in one of the fimbrial gene clusters, the *mad* mutants were able to hemaglutinate blood cells and form a biofilm on an abiotic surface (data not shown), indicating that *mad fimbriae* are not essential to these functions. Expression of *mad* fimbriae was detected in Grace's Insect media, and fimbriae were observed by transmission electron microscopy, but no apparent

differences between wild-type and mutant fimbriae were seen. In summary, we have identified the essential role of the putative *mad* fimbriae for symbiont transmission, specifically adherence to the maternal intestine of *H. bacteriophora.*

INTRODUCTION

The Gram-negative bacterium *Photorhabdus luminescens*, of the family Enterobacteriaceae, is symbiotically associated with the nematode *Heterorhabditis bacteriophora* (7). In this mutualistic relationship, the nematodes require the bacteria for growth, reproduction, and insect killing, while the bacteria require the nematode host as a vector for transmission to an insect prey (5, 11). Together, *P. luminescens* and *H. bacteriophora* are able to infect and kill a large variety of insect larvae (18, 22). In soil, *H. bacteriophora* persists as the developmentally arrested infective juvenile stage, which transmits *P. luminescens* cells in its intestine to insect hosts (14). Upon encountering insect prey, *H. bacteriophora* infective juveniles (IJs) migrate into the insect's hemocoel (blood cavity) either by means of natural openings, such as the mouth or anus, or by penetrating the outer cuticle (2).

Within the insect hemocoel, IJs regurgitate their symbiotic bacteria into the hemolymph, thereby infecting and killing the insect within 24-48 hours (6, 16). Within the insect's hemolymph, *P. luminescens* produce and secrete a large variety of toxins, antibiotics, antifungal compounds, nematicidal compounds, and enzymes, which function in the protection, preservation, and degradation of the insect cadaver (9, 23). Developmentally arrested IJs exit diapause and resume development within this nutritionally favorable environment and become egg-laying hermaphroditic adults (16). Eggs deposited into the insect cadaver develop into males, females, or hermaphrodites

(7). After several generations, eggs are no longer laid into the environment but instead hatch and develop into IJs inside the body of adult hermaphrodites and females – a process known as *endotokia matricida* (7). While developing within the maternal body cavity, pre-IJ's are recolonized by a single *P. luminescens* bacterium while feeding on the mother's body contents and leave the insect cadaver to actively hunt for another insect host (4). While they can feed on some other bacterial species, *H. bacteriophora* are highly specific as to the species of bacteria with which they associate (3, 10). In fact, when the nematodes are given a mixture of symbiotic and non-symbiotic bacteria, only the symbiotic bacteria will be transmitted to IJs (7).

In order to adapt to changing environments, some bacteria may undergo phase variation, which is a high-frequency ON ↔ OFF switching of phenotype expression (15). In *P. luminescens*, the switch from primary to secondary phenotypic variants occurs at much lower frequency and does not appear to be reversible (17). Only primary phenotypic symbionts are able to colonize *H. bacteriophora* and support nematode reproduction and development (12). Secondary phenotypic *P. luminescens* variants also lose some of the other important characteristics, such as inclusion bodies, antibiotic production, pigmentation, and bioluminescence, which are normally found in primary bacteria (12). While phase variation affects a large number of phenotypic characteristics, antigenic variation targets expression of surface organelles. For example, expression of *fimA* (which encodes the major structural subunit of pili in *Escherichia coli*) is regulated by inverting a region containing the promoter (1). *Photorhabdus luminescens* putative fimbrial gene cluster VI, which has a recombinase homologous to that found in the *fim* operon in *E. coli*, could also be regulated by such an invertible switch.

Bacterial attachment to a eukaryotic host cell is often the initial step in the colonization process of many bacteria (26). Attachment by means of pili or fimbriae is a common mechanism found in many species of Gram-negative and some Gram-positive bacteria (13, 25). The nematode symbiont *P. luminescens* has eleven gene clusters that are predicted to encode a variety of proteins related to known pili and fimbriae (8). This rather large number of gene clusters devoted to the biosynthesis of pili and fimbriae is uncommon in bacteria. Perhaps, symbiotic bacteria require specialized pili that allow them to adhere to their proper host in addition to common major types of fimbriae and pili.

In this study, we describe the identification of a *P. luminescens* putative fimbrial locus, *mad* (**m**aternal **a**dhesion **d**eficient), specifically required for adherence to the maternal posterior epithelium in *H. bacteriophora*, the first step in symbiont transmission.

MATERIALS AND METHODS

Note: Images in this thesis are presented in color.

Bacterial strains and culture methods

Photorhabdus luminescens and *P. temperata* were cultured in liquid PP3S media (2% Proteose Peptone #3 [Difco, Sparks, MD] containing 0.5% NaCl , and Grace's Insect Media (Invitrogen, Carlsbad, CA). Agar (1.5%), gentamicin (0.75 μg/mL), streptomycin (40 μg/mL), zeocin (50 μg/mL) and kanamycin (3.75 μg/mL) were added when required. *E. coli* strains were grown in Lysogenic Broth (LB) containing 5g/L NaCl. Agar (1.5%), gentamicin (Gm, 5 μg/mL), ampicillin (Amp, 50 μg/mL), zeocin (Zeo, 50 μg/mL), and diaminopimelic acid (DAP, 300 μg/mL) were added when

Table 2-1: Strains and plasmids used in this study.

Strain or Plasmid	Characteristic	Source
Nematodes		
Heterorhabditis bacteriophora TT01, inbred line M31e	inbred (self-fertilized for 13 times)	(Ciche and Sternberg)
Bacteria		
Photorhabdus luminescens subsp. *laumondii* TT01	wild-type (primary phase)	Nematode host
P. luminescens subsp. *laumondii* TT01-GFP	labeled with Tn*7*-GFP	(Ciche)
Photorhabdus temperata NC1	wild-type (primary phase)	ATCC29304
P. temperata NC1-GFP	labeled with Tn*7*-GFP	(Ciche)
TRN16	Transmission defective *P. temperata* NC1-GFP	(Ciche)
Escherichia coli BW29427	*dap* auxotroph	(W. Metcalf)
E. coli BW29427+ pURE10	mini-HimarGm	(D. Lies/D. Newman)
TransforMax EC100D *pir-116*	electrocompetent *E. coli*	EPICENTRE Biotechnologies
Plasmids		
pURE10	mini-HimarGm	(D. Lies/D. Newman)
pMadA-J	TOPO XL cloning vector, Zeo^r	Invitrogen
pMadHIJ	TOPO XL cloning vector, Zeo^r	Invitrogen

required. Freezer stocks were prepared by combining 5% DMSO with bacterial culture in PP3S and stored at -80 °C.

Axenic nematode stock

Nematodes of the inbred *H. bacteriophora* strain M31e were grown on lawns of the GFP-labeled **tr**a**n**smission mutant (TRN) 16, totally defective in transmission, on NA-cornoil (8 g of Nutrient broth, 15 g of agar, 12 mL of cornoil [Mazola] per liter) split plates for about 14 days. Then, Ringer's solution (100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES pH 6.9) was added to the empty half of the plate to collect the emerging IJs, which were harvested by centrifugation (1,200 rpm for 1 min.). The IJs were surface sterilized in 1% bleach (6.15% NaOCl) for 5 min., washed three times with Ringer's, and then stored in 10 mL of Ringer's solution in tissue culture bottles. Axenic stocks were tested for sterility by removing and disrupting a 45 µL sample using a motorized tissue grinder (Kontes Glass Co., Vineland, NJ). The ground sample was plated on PP3S and incubated at 28°C for 2 days to verify that the nematode stock was sterile. Antibiotics were added at the following concentrations: 100 µg/mL streptomycin, 100 µg/mL ampicilin, 30 µg/mL kanamycin, and 10 µg/mL gentamicin. The nematode stocks were also inspected under a fluorescent MZ16F stereomicroscope (Leica Microsystems, Wetzlar, Germany) to verify that no fluorescent bacteria were present in the intestines of the nematodes.

Adherence assay

Liquid cultures of *P. temperata* TRN16 (labeled with GFP) were spread on NA-cholesterol (recipe for NA as described above plus 10 mg/mL cholesterol) plates and incubated at 28°C for 48 hours. Axenic M31e nematodes were added to the TRN16

plates, incubated at 28°C, and after 16 hours transferred to plates containing *P. luminescens* TT01 Tn7GFP or mutant bacteria (labeled with GFP) for further incubation for 24 hours. Finally, the nematodes were transferred to plates with unlabeled TT01 and incubated for 4 hours to allow the nematodes to clear transient symbionts from their intestine before assaying for the presence of adherent *P. luminescens* using a fluorescent compound microscope (Leica DM5000).

Transposon mutagenesis and mutant screening

Photorhabus luminescens strain TT01 Tn7GFP, *P. temperata* strain NC1 Tn7GFP, and *E. coli* strain BW29427 donor cells containing pURE10 were grown overnight. 1/100 of the volume from each culture was transferred to fresh media and grown to an OD_{600} of 0.6. Cells were harvested by centrifugation, washed three times, and resuspended in 500 µl LB DAP. The recipient and donor suspensions were combined, centrifuged again, resuspended in 50 µl LB DAP, and plated on LB DAP. After incubation for 8 hours, the cells were washed off the plates with LB, centrifuged, washed two times, and resuspended in 1.5 mL of LB. 100 µl of cells were plated on LB Gm and incubated. Isolated colonies were picked from conjugation plates with sterile toothpicks, transferred to PP3S Gm, and incubated at 28°C for 48 hours. Each colony was subcultured in disposable 5 mL Falcon tubes (12x75 mm polystyrene) containing 250 µL of PP3S Gm. After incubation at 28°C for 48 hours, 50 µL of each liquid culture was spread to a lawn on to a NA-cornoil Gm plate. After incubation at 28°C for another 48 hours, 10 µL of nematode stock (M31e TRN16), washed three times with sterile 0.85% saline solution, was placed on each plate and incubated at 28°C for 10-12 days or until IJs migrated and became trapped in the condensation droplets present lids. IJs were

checked for colonization (GFP present or absent) under the fluorescent stereo microscope. Potential mutants were plated on PP3S plates.

Verification of mutant phenotype

Liquid cultures for each potential mutant were prepared and spread on NA-cholesterol plates. 10 µL of nematode stock (M31e TRN 16), washed three times with sterile 0.85% saline solution, was added to each plate and incubated at 28°C for 10-12 days or until IJs appeared in the condensation on the lid. On average, ~5000 IJs were checked for colonization (GFP present or absent) for each potential mutant using the fluorescent stereo microscope and the percentage of transmission efficiency (% colonized/total) determined.

Transposon retrieval and sequencing

The HimarGm transposon contains an R6Kγ origin of replication allowing direct retrieval of the transposon along with flanking DNA in strains expressing the pi protein. To retrieve the HimarGm, genomic DNA was purified using the DNeasy Tissue Kit (Qiagen, Valencia, CA) from verified mutant strains grown in 3 mL of Grace's Insect Media incubated at 28°C overnight. Five to 20 µg of genomic DNA was digested with *Sph1* restriction enzyme (New England Biolabs, Ipswitch, MA) to release the region of the genomic DNA containing the transposon plus DNA flanking the insertion. Linear digested DNA fragments were ligated with T4 DNA Ligase to construct circular DNA (New England Biolabs). The circular DNA was concentrated by isopropanol precipitation and resuspended in 10 µL ddH_2O. The ligation product was electroporated into TransforMax EC100D *pir-116* electro-competent *E.coli* cells (EPICENTRE Biotechnologies, Madison, WI) using a Gene Pulser Xcell Electroporation System

(BioRad, Hercules, CA).

After electroporation, *E. coli* cells were recovered in SOC broth, harvested by centrifugation, resuspended, and plated on LB Gm. Plates were incubated at 37°C overnight. Isolated colonies of transformants were grown overnight in liquid LB Gm. Plasmid DNA was extracted with Qiagen QIAprep Spin Miniprep Kit, digested with *Sac I* restriction enzyme to release a 1 kb fragment within the HiMar region of the transposon (New England Biolabs), and separated on 1% Agarose (Invitrogen). Plasmids that showed 1 kb fragments were submitted for sequencing. For sequencing, uncut plasmid DNA was combined with MarOut primer (5'-caagcttgtcatcgtcatcc-3') or GmOut primer (5'-cggtaaattgtcacaacgcc-3'). Sequencing was performed at the Michigan State University Research Technology Support Facility. Sequenced plasmids were analyzed with *coli*BLAST (http://colibase.bham.ac.uk/) to determine homologies to *P. luminescens* TT01 genes.

Complementation assay

Two regions of the *mad* cluster were amplified with an XL PCR kit (Invitrogen): *madA* through *madJ* (Fim Forward 5'-tgacagttaggaccattttgacaattg-3' and SpoT Reverse 5'-atccggctcacgactaaagaccgt-3'), and *madH* through *madJ* (Ush Forward 5'-acggccgtttacttgttgtcgttg-3' and SpoT Reverse). XL PCR was performed by combining 70-80 ng of genomic DNA with 2 µL of rTh DNA polymerase, followed by denaturation at 95°C for 1 minute. Then, 20 µL 3.3X XL PCR buffer, 8 µL 10 mM dNTP mix, 4.8 µL $Mg(OAC)_2$, 1.3 µL each of forward and reverse primers, and ddH_2O for a final volume of 50 µL. Thermocycler was set for 93°C for 1 minute, 30 cycles of 93°C for 1 minute, 57°C for 1 minute, 66°C for 15 minutes, and finally 72°C for 10 minutes. Fragment sizes

were checked by agarose gel electrophoresis.

The PCR products were cloned into the Topo XL PCR vector (Invitrogen) as per manufacturer's instructions. The vector was electroporated into TransforMax EC100D *pir-116* electro-competent *E.coli* cells (EPICENTRE Biotechnologies) and correct gene orientation and expression was tested by restriction endonuclease digestion. Liquid PP3S was inoculated with a TRN mutant overnight starter culture and grown to an OD_{600} of 0.6. The flasks were placed on ice for 20 minutes, and the cells harvested by centrifugation. The bacterial pellet was washed twice with ice cold 10% glycerol, centrifuged, and resuspended in 1 mL of ice cold 10% glycerol. Most of the supernatant was removed by centrifugation, and the bacteria were aliquoted into clean 1.7 mL microcentrifuge tubes so that each tube contained about 35 µL. Each aliquot was mixed with 1.5 µL of the constructed Topo XL PCR vector (Invitrogen) containing either the entire *P. luminescens* TT01 gene cluster VI (*madA* through *madJ*) or a portion (*madH* through *madJ*) and electroporated as described above. After electroporation, the cells were recovered in SOC broth, harvested by centrifugation, and plated on PP3S Zeo. Plates were incubated at 28°C for two days or until colonies appeared. Transformants were grown in liquid PP3S Zeo and plated on NA-cholesterol Zeo plates. At the same time, liquid cultures of *P. luminescens* TT01, TT01 Tn7GFP, and TRN16 were plated on NA-cholesterol plates and incubated. Transformants were tested in *H. bacteriophora* following the adherence assay described above.

Inverted repeat analysis

The DNA sequence upstream of *madA* was searched for the presence of inverted repeats using "EMBOSS einverted"

(http://bioweb.pasteur.fr/seqanal/interfaces/einverted.html). Putative promoter sites were predicted with "BPROM" (http://www.softberry.com). Primers were designed to amplify a 1 kb region including the inverted repeat (Figure 2-1 A; Method adapted from 1): fimIR forward 5'-taggccatcgttttgggtag-3' and fimIR reverse 5'-actcgtgcatcacgttttga-3'. A *SfcI* restriction site located within the inverted repeat was used to determine orientation (ON or OFF). After PCR amplification of *P. luminescens* strain TT01 Tn7GFP (primary and secondary phenotypic variants) and TRN26-25 (primary) genomic DNA, and bacterial genomic DNA extracted from boiled nematodes (after 24 and 48 hours), the PCR product was digested with *SfcI* and separated on a 2% agarose gel. In the putative ON position, fragments of 925 bp and 128 bp were expected (Figure 2-1 B). In the putative OFF position, fragments of 753 bp and 300 bp should be seen (Figure 2-1 B).

In addition, PCR amplification was also performed using colonized *H. bacteriophora* to determine the state of the switch within *P. luminescens* in the nematode intestine. Nematodes were grown on TT01 Tn7GFP for 24 and 48 hours. The nematodes were picked off the plates and washed in sterile saline. Then, individual *H. bacteriophora* were placed in PCR tubes containing ddH_2O and heated at 95°C for 10 minutes. Axenic *H. bacteriophora* IJs were heated alongside as a negative control. After a brief spin, the supernatant was used as template for PCR amplification.

Figure 2-1: A) Nucleotide sequence upstream of *madA* (capital letters) that contains the inverted repeat (black boxes), primer sites (underlined), *SfcI* recognition site (grey box), and the possible promoter site, as indicated by the presence of a -10 and -35 box. The start of the *madA* open reading frame is indicated by capital letters. The start codon is highlighted by a box. B) Size of inverted repeat PCR fragments after digestion with *SfcI*. Fragment sizes are different depending on whether the repeat is in the ON or OFF postion. (Method adapted from 1)

A)

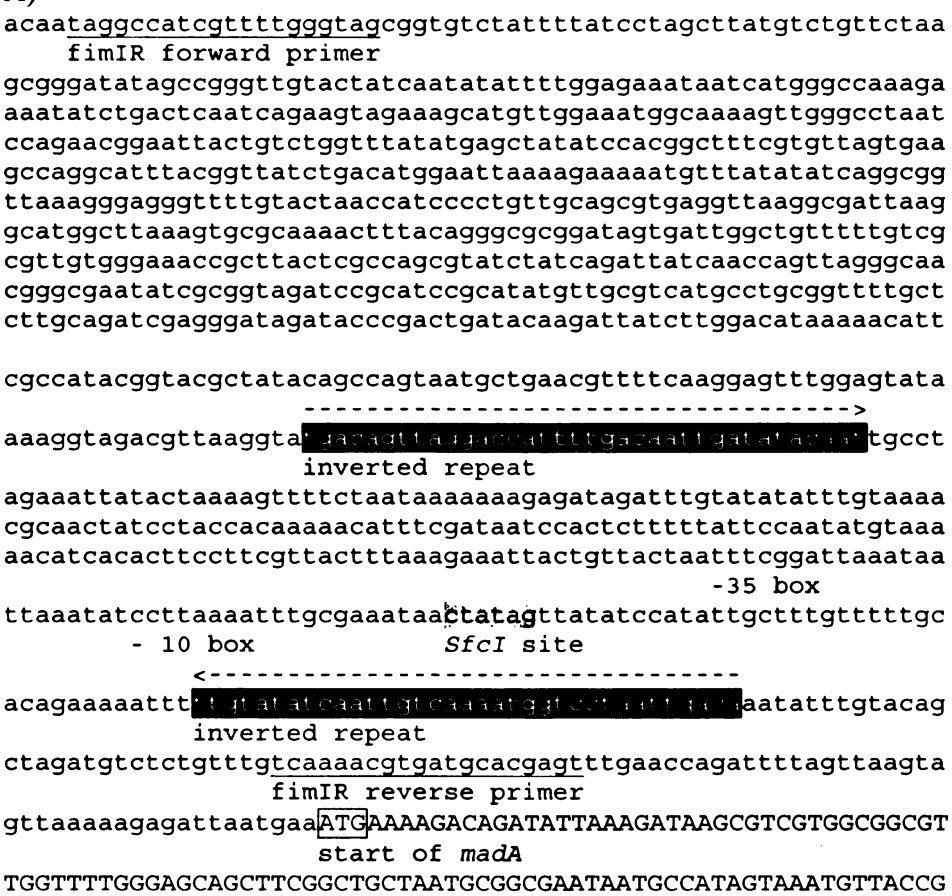
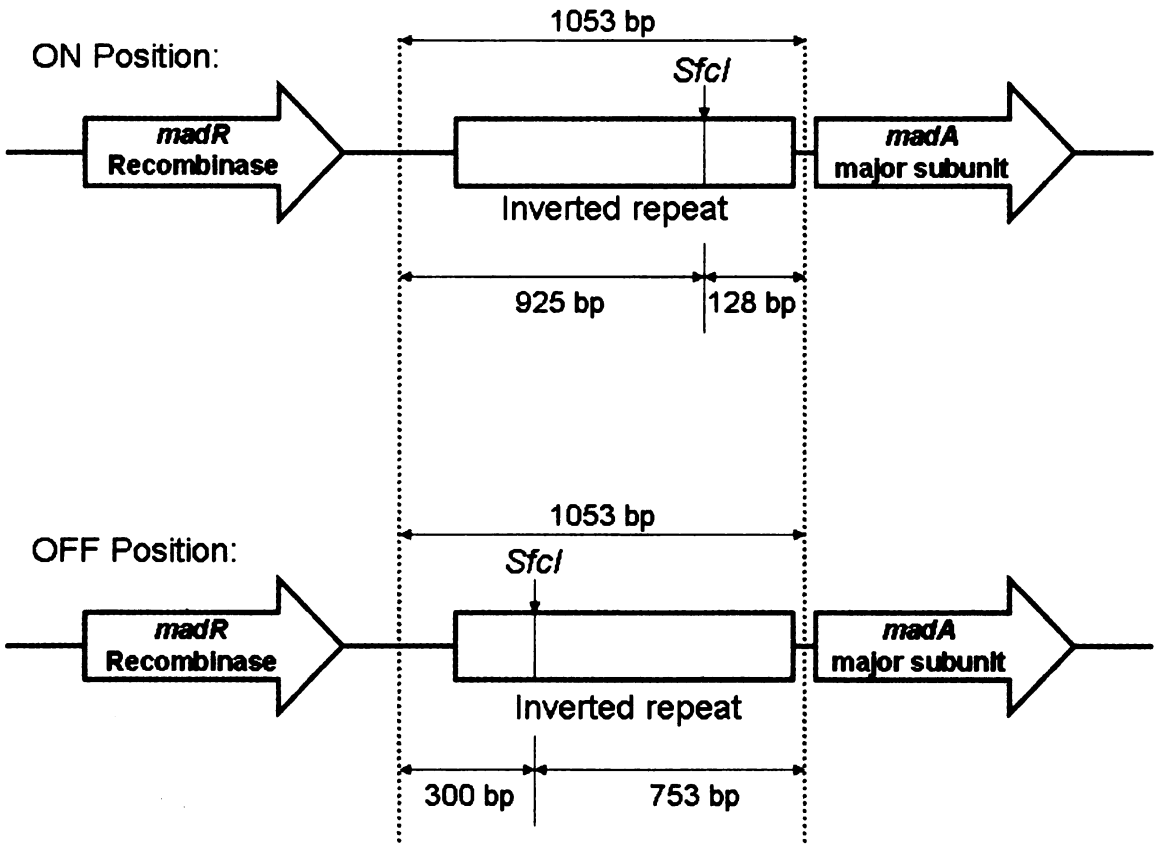


Figure 2-1 (continued)

B)



RT-PCR for major fimbrial subunits

RNA was isolated from bacteria grown in Grace's Insect medium liquid cultures. Bacteria were harvested by centrifugation, frozen in liquid nitrogen, and resuspended in Trizol. After addition of chloroform and a brief incubation time, the tubes were centrifuged and the resulting aqueous, colorless, RNA containing phase was transferred to a clean tube before addition of isopropanol. After centrifugation, the RNA pellet was washed twice with ice cold 70% ethanol (diluted in DEPC H_2O) and centrifuged. After air drying, the pellet was resuspended in 10 µL DEPC H_2O, and RNA yield and quality

was measured with a NanoDrop ND-1000 (NanoDrop Inc., Wilmington, DE). RNA was treated with DNAseI and reverse transcribed with Thermoscript Reverse Transcriptase (Invitrogen). The resulting cDNA was amplified in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Primers used were as follows:

*madA*for 5'-gtatttgccgcgtttttgtt-3', *madA*rev 5'-gccgaagcagacgaaactac-3'

*mrfA*for 5'-gtcacggaacggtgactttt-3', *mrfA*rev 5'-gtcagctggaagttggcaat-3'

*plu0779*for 5'-tgctcttaagcatggggatg-3', *plu0779*rev 5'-aaagctaaccgcatcattgg-3'

*plu0786*for 5'-gatgtgagtgccggtacctt-3', *plu0786*rev 5'-ggagtctgctgcttttaccg-3'

*plu0418*for 5'-ccctagcagcaggttctgtc-3', *plu0418*rev 5'-ccccgtcaccttgtaagaaa-3'

*plu0507*for 5'-gtgcagtcactgatgccact-3', *plu0507*rev 5'-atcaccagcgcctactttgt-3'

*phfS*for 5'-tttggcttctttagtgacggta-3', *phfS*rev 5'-tgaaattcatagtggcggtgt-3'

*plu2159*for 5'-tatcatcgcgaatgcctgta-3', *plu2159*rev 5'-atcgtaaaattggcgactgc-3'

*pilL*for 5'-ctgtgtgataccaccgcaac-3', *pilL*rev 5'-ctatcggtaccgttgccact-3'

*plu1732*for 5'-tcggaaatctcaaccgaatc-3', *plu1732*rev 5'-aaaccagcgtcacggtattc-3'

Negative controls included DNaseI treated but not reverse transcribed RNA and ddH_2O. Positive control used was genomic DNA from *P. luminescens* TT01 and TRN26-25. PCR products were separated on a 1% agarose gel to check for presence or absence of bands.

Transmission Electron Microscope Imaging

Cultures of TT01 Tn7GFP and TRN26-26 were grown in liquid Grace's Insect media in a 28°C shaker overnight. The cultures were diluted 1:1 with fresh Grace's Insect media and transferred to a 300 mesh nickel grid, coated with formvar, by submersing the grid into the diluted culture to collect bacteria. Negative staining was performed with 4%

ammonium molybdate. Grids were examined with a JEOL 100CX transmission electron microscope at the MSU Center for Advanced Microscopy.

RESULTS

Photorhabdus temperata *and* P. luminescens *mutant screening in* H. bacteriophora

In order to determine which genes are required for transmission of *Photorhabdus* spp. to *H. bacteriophora*, the bacterial mutants isolated during the transposon mutagenesis where screened for their inability to colonize the intestine of *H. bacteriophora* IJs. Of the ~8,000 screened mutants, 30 mutants were isolated which were transmission defective. Transmission was determined by the presence or absence of GFP throughout the IJ intestine when observed under a fluorescent microscope. While these mutants were not able to be transmitted, they still displayed other wild-type primary phenotypic characteristics such as pigmentation, bioluminescence, and colony morphology, when observed under a dissecting microscope.

Twelve of the *Photorhabdus* spp. **tr**a**n**smission (TRN) mutants had transposon insertions in genes homologous to those of *P. luminescens* fimbrial gene cluster VI (Figure 2-2), comprised of genes *madR* through *madJ*, which is predicted to encode putative fimbrial proteins assembled by an usher-chaperone pathway. The defective *mad* genes that resulted in a transmission phenotype were the major structural subunit *madA* (TRN359), the three chaperones *madB,F, and G* (TRN 5-60, TRN10-245, TRN 17-60, and TRN23-30), the putative adhesion subunit *madE* (TRN16-158, TRN19-124, and TRN 24-55), and the outer membrane usher *madH* (TRN583 and TRN18-110; Figure 2-2). Two genes encoding proteins of unknown function were also mutated: *madI* (TRN22-68) and *madJ* (TRN26-25; Figure 2-2). These fimbrial TRN mutants are defective in the

initial adherence event that takes place in recovering IJs 12 hours after regurgitation. All fimbrial TRN mutants had 0% transmission efficiency (>5,000 *H. bacteriophora* IJs assayed for transmission of any symbiont bacteria), except for TRN22-68 (46.2%) and TRN24-55 (2.3%; Table 2-2).

A comparison of *mad* genes to their *E. coli* homolog revealed significant homologies of three *mad* genes (*madB,F, and G*) with *E. coli* chaperones (encoded by *fimC*; Table 2-3). Significant homology was also seen between *madH* and the outer membrane usher protein in *E. coli* encoded by *fimD* (Table 2-3). Both *madA* and *madD* were homologous to a minor fimbrial subunit FocF of *E. coli* (Table 2-3), No significant homologies were seen for the two *mad* genes with unknown function, *madI* and *madJ* (Table 2-3), but *madE* was homologous to an adhesion protein when the retrieved plasmid of TRN16-158, TRN19-124, and TRN24-55 was compared with *P. luminescens* TT01 using *coli*BASE. The recombinase MadR was similar to the recombinase FimB in *E. coli.* Overall, *mad* genes were predicted to encode fimbriae similar to Type I fimbriae in *E. coli* encoded by the *fim* operon (Table 2-3).

The remaining TRN mutants had transposon insertions in 18 different loci, including *sspA* (stress response), *sctP* (type III secretion), *wzc* (capsule secretion and polymerization), *nhaA* (Na^+/H^+ antiporter), *livM* (leucine, isoleucine, and valine transporter), and *abgR* (p-aminobenzoyl-glutamate catabolism). The role of these genes and others in symbiont transmission is currently being investigated by the Ciche Lab (Michigan State University, Department of Microbiology and Molecular Genetics).

Figure 2-2: Locations of transposon insertions in the *P. luminescens* TT01 fimbrial gene cluster VI. Arrows do not indicate exact insertion site. This gene cluster is predicted to encode Type I usher-chaperone fimbriae. All TRN mutants are *P. temperata* NC1, except for TRN26-25, which was made from *P. luminescens* TT01. It is possible that the NC1 gene cluster is slightly different from the same gene cluster in TT01.

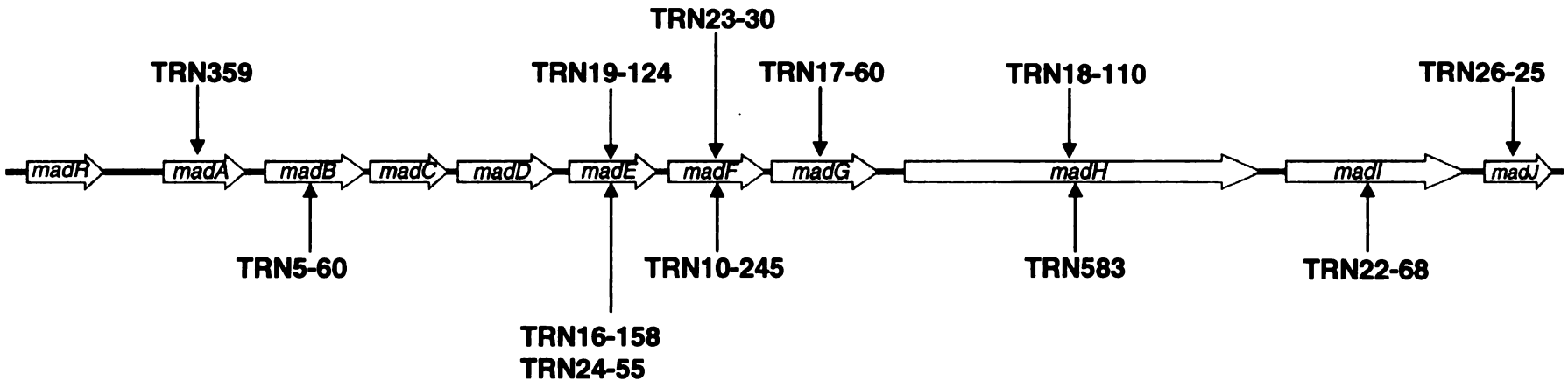


***Table 2-2*:** Transmission efficiencies of *P. temperata* NC1 and *P. luminescens* TT01 TRN mutants (as determined during the mutant screening) and approximate transposon insertion sites (determined with *coli*BLAST).

TRN mutant	*Insertion in P. luminescens gene*	*Gene size (bp)*	*Insertion site (nt)*	*% Transmission efficiency*
TRN359	*madA*	606	225	0
TRN583	*madH*	2631	1710	0
TRN5-60	*madB*	741	573	0
TRN8-17	n/d	n/d	n/d	0
TRN10-245	*madF*	720	717	0
TRN16-158	*madE*	657	n/d	0
TRN17-60	*madG*	795	n/d	0
TRN18-110	*madH*	2631	n/d	0
TRN19-124	*madE*	657	60	0
TRN22-68	*madI*	1326	102	46.2
TRN23-30	*madF*	720	n/d	0
TRN24-55	*madE*	657	636	2.3
TRN26-55 (TT01)	*madJ*	501	147-174	0

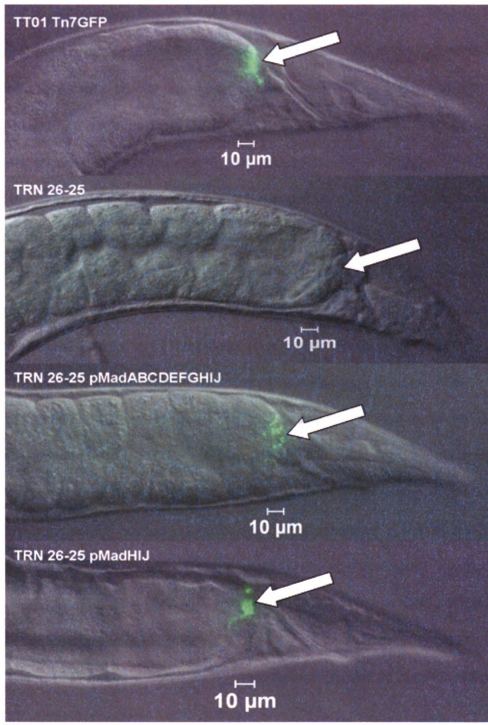
Table 2-3: Comparison of *P. luminescens mad* genes with their *E. coli* homolog and their proposed functions. E-values below 0.0 are considered significant.

P. luminescens Gene	Length in AA	E. coli Homolog	Proposed protein function	E-value	% Ident.	% Pos.
madR	188	*fimB*	Type 1 fimbriae regulatory protein	2.00E-63	59	76
madA	201	*focF*	F1C minor fimbrial subunit F precursor	4.00E-04	25	39
madB	246	*fimC*	Chaperone protein	5.00E-19	34	54
madC	190	*yadL*	unknown; hypothetical protein	0.047	22	38
madD	240	*focF*	F1C minor fimbrial subunit F precursor	4.00E-04	24	43
madE	218	*ycbQ*	Hypothetical fimbrial-like protein	0.13	19	42
madF	239	*fimC*	Chaperone protein	1.00E-32	35	53
madG	264	*fimC*	Chaperone protein	3.00E-18	28	49
madH	876	*fimD*	outer membrane usher protein	e-159	37	55
madI	441	none	unknown	n/d	n/d	n/d
madJ	166	none	unknown	n/d	n/d	n/d

TRN mutant complementation

After verifying *mad* gene orientation and expression of complementing plasmids in *E. coli*, three of the *P. temperata* NC1 TRN mutants in the *mad* cluster were transformed with either the complete (pMadA-J) or partial (pMadHIJ) gene cluster: TRN583 (transposon insertion in *madH*), TRN17-60 (*madG*), and TRN22-68 (*madJ*). None of these transformed mutants were able to colonize *H. bacteriophora* after 24 hours, as there was no GFP detected in the intestine. When the *P. luminescens* TT01 mutant TRN26-25 (transposon insertion in *madJ*) was complemented with the complete (pMadA-J) or partial (pMadHIJ) gene cluster, the bacteria were able to colonize *H. bacteriophora* again (Figure 2-3). As expected, wild-type strain TT01 Tn7GFP bacteria (positive control) were able to fully colonize *H. bacteriophora*, while untransformed TRN26-25 (negative control) was not able to initiate adherence and colonization (Figure 2-3). TRN26-25 complemented with pMadA-J or pMadHIJ were partially restored in the ability to adhere to the maternal intestine, and TRN26-25 pMadHIJ was better able to adhere to *H. bacteriophora* intestine than TRN26-25 pMadA-J. In addition, IJs developed from nematodes grown on TRN26-25 pMadA-J and TRN26-25 pMadHIJ, but these IJs were not colonized by bacteria.

Figure 2-3: *Photorhabdus luminescens* TT01 Tn7GFP, TRN26-25, and transformed TRN26-25 bacteria in 44-hour post-IJ *H. bacteriophora*. Nematodes were recovered on TRN16 for 16 hours, transferred to either TT01 Tn7GFP, TRN26-25, TRN26-25 pMadA-J, or TRN26-25 pMadHIJ for 24 hours, and placed on unlabeled TT01 for 4 hours. White arrows point to rectal gland cells that have been invaded (GFP present) or not (no GFP).

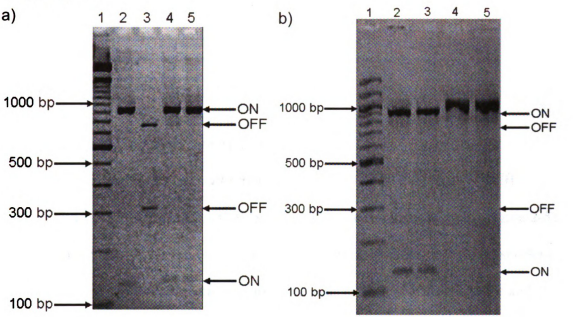


Inverted repeat switch

Analysis of the upstream region of *madA* revealed the presence of an inverted repeat (IR). The IR is 35 bp long, matches at 33 bp (94% complementarity) with 0 gaps, and has a 31.4% GC content (Figure 2-1 A). Analysis of the same region (Length of input sequence: 1121 bp; threshold for promoters: 0.20; scores above 0.20 are significant) also yielded a predicted promoter site within the IR, with a -10 box at position 809 (Score: 68) and a -35 box at position 786 (Score 13; Figure 2-1 A). The presence of an IR and *madR* recombinase just upstream of *madA* suggested that gene cluster VI might be regulated by phase variation under different growth conditions by inverting the promoter. To test this, the orientation of the *mad* promoter was analyzed in primary phenotypic variants which are transmitted in IJs and secondary phenotypic variants that are not.

In *P. luminescens* primary phenotypic variants (TT01 Tn7GFP, TRN26-25, and TRN26-25 pMadABCDEFGHIJ 1°) the switch was primarly in the predicted ON postition (Figure 2-4), as evidenced by *SfcI* restriction endonuclease treated PCR products of 925 bp and 128 bp. However, a small percentage (<5%) of the bacterial population seemed to be in the "OFF" position, since very faint bands were observed at 750 and 300 bp (data not shown). In TT01 Tn7GFP secondary phenotypic variants (2°) the switch was in the OFF position, as evidenced by fragment sizes of 750 bp and 300 bp only detected (Figure 2-4). In addition, PCR amplification of *P. luminescens* genomic DNA recovered from boiled worms indicated that the switch is in the "ON" position in *P. luminescens* within the nematode intestine (Figure 2-4). These results indicate that the IR switch is ON in 1° *P. luminescens* (from liquid cultures and boiled *H. bacteriophora*) and OFF in 2° *P. luminescens* (from liquid culture).

Figure 2-4: *SfcI* digested inverted repeat PCR products from *P. luminescens* TT01 Tn7GFP and TRN26-25 colonies (a) or boiled *H. bacteriophora* (b) on 2% agarose gel. Lane contents in a) are: 1) 100 bp ladder, 2) TT01 Tn7GFP 1°, 3) TT01 Tn7GFP 2°, 4) TRN26-25 1°, and 5) TRN26-25 pMadA-J 1° (4). Lane contents in b) are: 1) 100 bp ladder, 2) TT01 Tn7GFP from 24 hr. post-IJ *H. bacteriophora*, 3) TT01 Tn7GFP from 48 hr. post-IJ *H. bacteriophora*, 4) same as (1) but uncut fragment, and 5) same as (2) but uncut fragment. ON position: 925 bp and 128 bp fragments. OFF position: 753 bp and 300 bp fragments.



RT-PCR for major fimbrial subunits

To determine if, in fact, the promoter switch is in the "ON" position of primary phenotypic *P. luminescens*, transcription of *madA* was analyzed by RT-PCR. Since the IR switch was ON in *P. luminescens* TT01 and TRN26-25 1°, *mad* fimbriae should be expressed in both. Expression of *mad* fimbriae was tested by RT-PCR. The resulting expression profiles of major fimbrial subunits were identical *in P.* luminescens TT01 and TRN26-25 (Table 2-4). Both showed expression of *madA*, as well as all other major fimbrial subunits, except for *pilL* which was not expressed (Table 2-4).

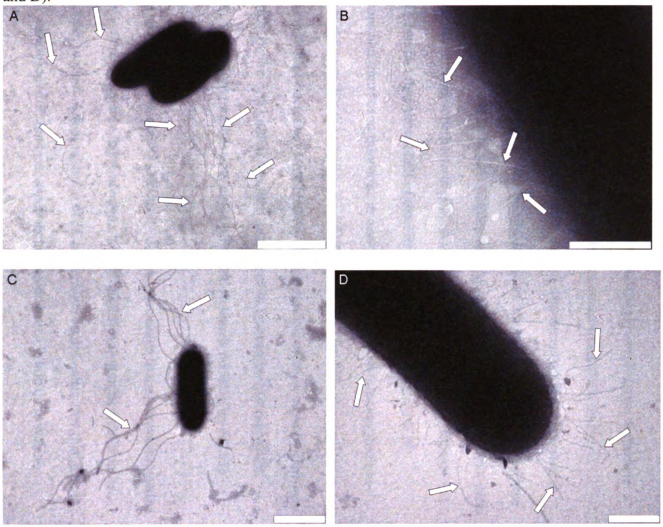
Table 2-4: Expression of major fimbrial subunits in 1° *P. luminescens* TT01 Tn7GFP and TRN26-25 as measured by RT-PCR.

Primer	TT01 Tn7GFP	TRN26-25
madA	+	+
mrfA	+	+
plu0779	+	+
plu0786	+	+
plu0418	+	+
plu0507	+	+
phfS	+	+
plu2159	+	+
pilL	-	-
plu1732	+	+

Transmission electron microscopy (TEM)

We attempted to detect *mad* fimbriae using TEM of *P. luminescens* TT01 Tn7GFP and TRN26-25. Surprisingly, both strains were indistinguishable (Figure 2-5). Both had long peritrichous flagella (Figure 2-5). Under higher magnification, shorter pili (or fimbriae) were found covering the entire bacterial surface of both wild-type and mutant. This suggests that fimbriae other than *mad* are expressed on plates or in liquid cultures. Expression of *mad* fimbriae is possibly restricted to the intestine of *H. bacteriophora*, or they might be indistinguishable from other fimbriae that are present.

Figure 2-5: TEM images of *P. luminescens* TT01 Tn7GFP (A and B) and TRN26-25 (C and D) on a 300 mesh Nickel grid coated with Formvar. White arrows point to flagella (A and C) or fimbriae (B and D). White bars represent 2 µm (A and C) and 500 nm (B and D).



DISCUSSION

Specialized fimbriae and pili play a key role in the attachment of human pathogens to their proper host. For example, *Salmonella enterica* serovar Typhi has IVB pili that were found to be essential for pathogenesis in humans (21). *Photorhabdus temperata* possesses an *mrf* operon encoding mannose-resistant fimbriae that are expressed prior to insect lethality but their exact role during insect pathogenesis is unknown (20). In *Vibrio cholerae*, the flexible NAGV14 pili were shown to mediate adhesion, allowing the bacteria to adhere to rabbit intestinal tissue (26). In *Ps. aeruginosa*, only *cup* fimbriae are involved in surface attachment and are expressed in response to environmental signals indicating a favorable attachment site (19, 24). These examples demonstrate how pathogens employ specialized fimbriae to adhere to host cells, so it is possible that symbiotic bacteria possess similarly specialized means of host attachment. The fact that almost half of the TRN mutants unable to colonize *H. bacteriophora* had transposon insertions in one of the eleven *P. luminescens* fimbrial gene clusters suggests that the putative *mad* fimbriae encoded by gene cluster VI play a key role in initial host-bacteria interactions during the gut colonization process. The *mad* fimbriae might be the first kind of fimbriae discovered to be absolutely required for symbiosis and symbiont transmission, although further research needs to be done to support this hypothesis.

The complemented mutant TRN26-25 was able to adhere to the maternal intestine of *H. bacteriophora*. Because the *madJ* mutant (TRN26-25) was complemented in its ability to adhere to the maternal intestine of *H. bacteriophora*, it is clear that this gene is required for adherence of *P. luminescens* to its nematode host. While there was no

transmission of complemented TRN26-25 from mother to offspring, these findings still indicate the significance of these apparently specialized fimbriae in the colonization process. Since the transformed mutants had to be grown on media containing Zeocin, the presence of the antibiotic seemed to have a minor effect on nematode development (such as increased recovery time), which could explain why TRN26-25 pMadA-J and pMadHIJ did not show levels of colonization similar to wild-type. Also, it is very difficult to maintain plasmid selection inside the nematode intestine. As seen in this study, the complemented mutant TRN26-25 was able to adhere to the maternal intestine within 24 hours, but when *H. bacteriophora* were grown until IJs developed, those IJs were not colonized. The majority of the bacterial population, therefore, might have lost the plasmid within the nematode intestine, where antibiotic selection can no longer be maintained, and were no longer able to utilize the plasmid to make the fimbriae required for colonization and transmission to offspring.

Although *P. luminescens* and *P. temperata* are genetically very similar, and both can successfully colonize *H. bacteriophora*, the question remains why *P. temperata* mutants TRN583, TRN17-60, and TRN22-68 did not complement. Since both *Photorhabdus* species can colonize *H. bacteriophora*, they apparently produce similar fimbrial proteins that allow them to adhere to the nematode intestine and to be transmitted to offspring. While these fimbrial proteins might be very similar, they may adhere to different receptors within the nematode intestine. However, maintaining the plasmid within the nematode intestine continues to be a problem, even after successful complementation. In order to solve this problem, the mutations found during transposon

mutagenesis need to be reconstructed by making *P. luminescens* TT01 Tn7GFP knockouts by a single insertion of a gene deletion into the chromosome.

Phase variation results in a mixed population of phenotypes, which allows bacteria to quickly adapt to new environments (15). Populations of *P. luminescens* are no exception. After seven days of culturing on a plate, populations of *P. luminescens* were observed to consist of mostly primary phenotypic variants (1°), but a small group of secondary phenotypic variants (2°) was also present (data not shown). After 14 days of culturing on the same plate, the same population consisted entirely of 2° *P. luminescens.* With regards to the *mad* gene cluster, the IR switch was seen to be ON in 1° *P. luminescens* and OFF in 2°. *P. luminescens* TT01 Tn7GFP 1° and TRN26-25 1° expressed *mad* genes, as seen by RT-PCR, providing further evidence that the IR switch is ON. Secondary phenotypic variants of *P. luminescens* are not able to colonize *H. bacteriophora* (12). Bacterial genomic DNA isolated from nematode intestines revealed the IR switch to be ON within the nematode intestine, suggesting that this gene cluster is involved in the colonization process. Also, since the switch was ON in both TT01 Tn7GFP and TRN26-25, it suggests that the mutant is a primary phenotypic variant. In addition, TRN26-25 exhibited 1° characteristics, such as pigmentation, bioluminescence, and colony morphology when observed using a dissecting microscope. Therefore, the observed inability of this mutant to colonize *H. bacteriophora* is due to the gene disruption and suggests that TRN26-25 is a true mutant and not just a 2° functioning in a way similar to a mutant. The presence of an inverted repeat switch suggests that madA and perhaps other genes critical for mad function might be regulated by the IR.

Differences in the amount of fimbriae present were seen during TEM imaging,

depending on what media *P. luminescens* was grown in. When bacteria were grown in PP3S, pili and fimbriae were sparse and mostly absent, even on the wild-type strain surface. When bacteria were grown in Grace's Insect media, pili and fimbriae were abundant and found on almost all (~95%) bacteria in a sample. Grace's Insect media is a rich media that mimics the environment found within an insect host, and nutrient availability may have stimulated production of fimbriae. Based on the expression results from the RT-PCR, there could be three reasons why there was no difference detected by TEM: 1) *mad* fimbriae were present in both, wild-type and mutant, but were non-functional, 2) *mad* fimbriae are transcribed but not made in Grace's Insect media, and 3) *mad* fimbriae were lost in the mutant but loss was not detectable. While the structural differences between *P. luminescens* wild-type and TRN26-25 pili were not obvious under the microscope, the differences could be found at a much smaller molecular level. Perhaps, the protein encoded by *madJ* (the defective gene in TRN26-25) has an essential accessory function, such as the correct assembly of fimbrial subunits, or attachment of the correct adhesin protein to the fimbrial tip and sides.

Since the *mad* proteins are very similar in size to each other, SDS-PAGE analysis would probably be very difficult. A better approach would be to align amino acid sequences of Mad proteins to each other and to other fimbrial proteins to detect unique residues only found in Mad proteins. These unique residues could be used to develop antibodies, which could be labeled with fluorescent probes and used in a Western blot that could detect which proteins are present or absent. Another approach could be to shear fimbriae off the bacterial surface and run the sample through a mass spectrometer. In order to test whether or not *mad* proteins are present during different growth

conditions, Western blots with labeled *mad* antibodies could also be done for *mad* proteins isolated from liquid culture, from nematodes at different developmental stages, and from insect larvae. Based on the results of this study, *mad* fimbriae possibly are expressed in L3 *H. bacteriophora* after regurgitation, when *P. luminescens* initiates adherence to the posterior intestine, and in pre-IJs that are colonized by adherence of a single *P. luminescens* cell to the pharyngeal-intestinal valve cells. Since the adherence of *P. luminescens* is specific to *H. bacteriophora*, *mad* fimbriae might be strain specific and arose by co-evolution.

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CHAPTER 3: SUMMARY AND FUTURE DIRECTIONS

This chapter is divided into two parts. Part I summarizes the findings presented in the chapters and appendices of this thesis. Part II makes suggestions for future research in addition to that presented in Chapter 2.

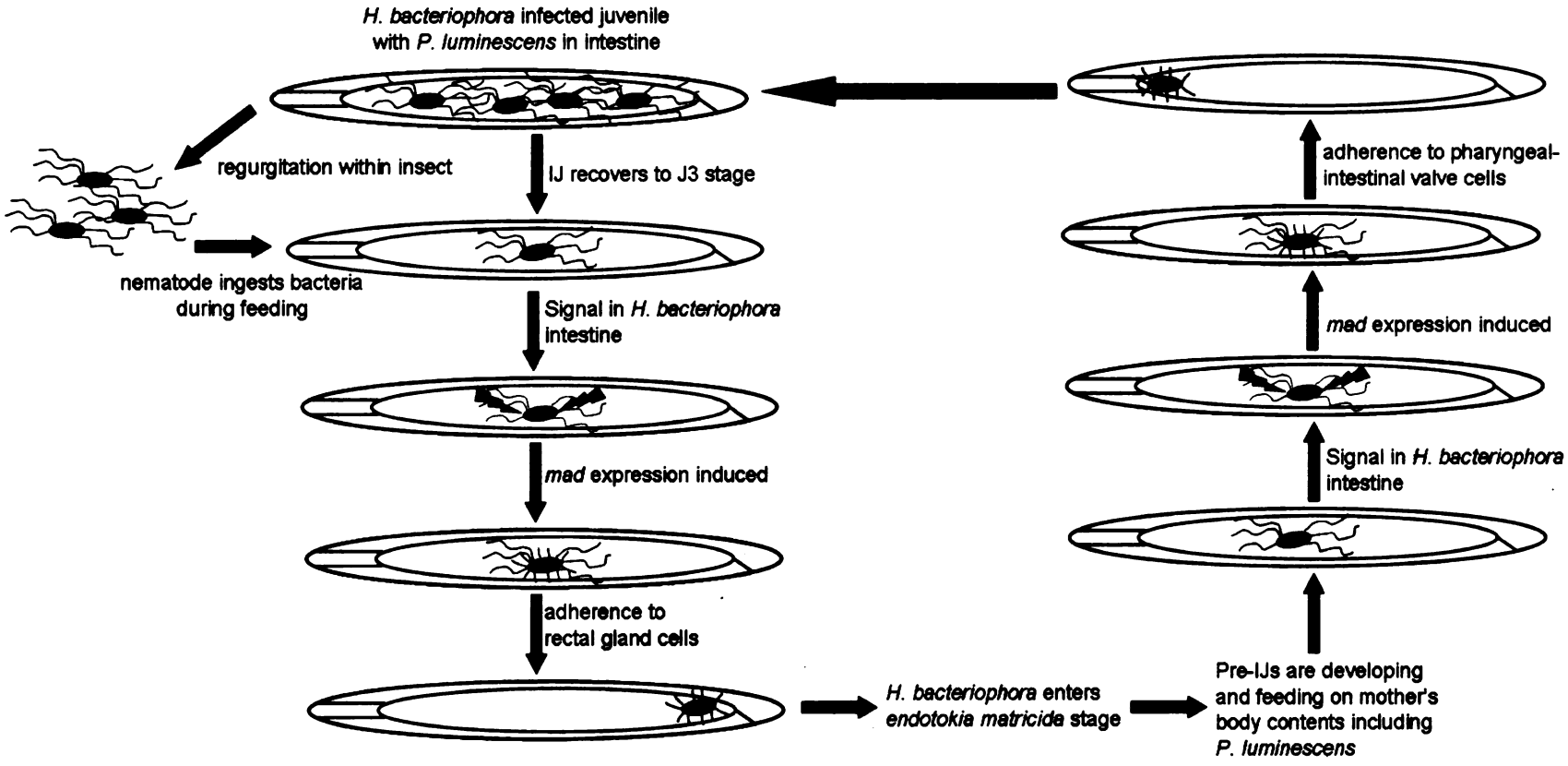
SUMMARY

The results of this study were able to answer some fundamental questions regarding the symbiotic relationship of *P. luminescens* with *H. bacteriophora*: 1) which genes are important in symbiosis? *Photorhabdus luminescens* with transposon insertions in the mad fimbrial gene cluster were not able to colonize *H. bacteriophora*. Therefore, *mad* fimbriae likely play an essential role during initial host-bacteria specific interactions in the nematode intestine colonization process. 2) Can these mutants colonize *H. bacteriophora* again when wild-type genes are supplied? TRN26-25, when complemented with the wild-type gene cluster, was able to adhere to *H. bacteriophora* maternal intestinal cells again. While adherence was only partially restored, and there was no transmission from mother to offspring, this finding supports the hypothesis that this particular type of fimbriae must be nematode-specific and important in the symbiotic relationship of *P. luminescens* with *H. bacteriophora*. 3) How are *mad* fimbriae regulated? The *mad* gene cluster in *P. luminescens* seems to be regulated by an IR switch that is ON in primary phenotypic variants grown on plates and from within the nematode intestine and OFF in secondary phenotypic variants grown on plates. Expression of *mad* genes was seen in *P. luminescens* primary phenotypic variants, further suggesting that the IR switch is in the ON position. Further research needs to be done to confirm that the *mad* gene cluster is an operon (see appendices). 4) Are other fimbriae present and functional in *mad* mutants? All TRN fimbrial mutants were able to form a biofilm on an abiotic surface (see appendices) and hemaglutinate blood cells (data not shown), suggesting that the other fimbrial gene clusters in *P. luminescens* are functional and are not involved in the colonization process. The absence of obvious structural differences,

as observed by TEM, suggests that differences between wildtype and mutant fimbriae are at a much smaller molecular level that need to be investigated.

In conclusion, I would like to present the following model depicting the hypothetical series of events that lead to adherence and transmission of *P. luminescens* in *H. bacteriophora* mediated by *mad* fimbriae (Figure 3-1). After regurgitation within the insect larvae, *H. bacteriophora* ingests *P. luminescens* cells while feeding on the insect body contents (Figure 3-1). An unknown signal within the intestine of *H. bacteriophora* might initiate expression of *mad* fimbriae, which allow the bacteria to adhere near the rectal gland cells (Figure 3-1). After several generations, *H. bacteriophora* no longer lays eggs into the environment, and IJs develop within the mother's body. While feeding on the body contents of the mother, pre-IJs ingest *P. luminescens* once again (Figure 3-1). After ingestion, an unknown signal might initiate expression of *mad* fimbriae, which allow the bacteria to adhere to the pharyngeal-intestinal valve cells (Figure 3-1). From there, *P. luminescens* recolonizes the entire intestine of *H. bacteriophora* (Figure 3-1).

Figure 3-1: A hypothetical model of adherence of *P. luminescens* to *H. bacteriophora* intestinal cells as mediated by *mad* fimbriae.



FUTURE DIRECTIONS

The results of this study were helpful to answer some of the fundamental questions associated with bacteria-nematode symbiosis, but there are a number of questions that still need to be answered. In order to further test the importance of *mad* fimbriae in colonization and transmission, one could run adhesion inhibition tests. There are two ways in which these tests could be conducted: 1) wild-type and mutant bacteria could be treated with anti-fimbriae antibodies before testing them in the nematodes, and 2) nematode intestines could be treated with purified *mad* fimbriae to block receptors before testing adherence of wild-type and mutant bacteria to the nematode intestine. In both cases, if *mad* fimbriae are important for colonization, wild-type bacteria should not be able to colonize the nematode intestine. The mutant bacteria act as a negative control.

In addition, it would be interesting to see if TRN fimbrial mutants could still be pathogenic in insects. For this purpose, one could inject serial dilutions of TRN fimbrial mutants liquid cultures into insect larvae, such as *Manduca sexta*, and determine insect survival curves and LD_{50}. If TRN fimbrial mutants are not pathogenic to insects, *mad* fimbriae might act as a virulence factor in insects.

Most importantly, it would be important to determine what surface receptors *mad* fimbriae bind to within the nematode intestine. There are several ways in which this question could be answered. For example, one could assemble a glycoarray containing a variety of sugars and probe the array with a solution of sheared *mad* fimbriae that have been labeled with a fluorescent probe. If fluorescence is detected for a certain sugar, that sugar might be the receptor to which *mad* fimbriae bind to on the surface of *H. bacteriophora* intestinal cells. Another way would be affinity chromatography, during

which labeled *H. bacteriophora* intestinal cells are run on a column of immobilized *mad* fimbriae. This would give some insights into what kind of nematode cells *mad* fimbriae can bind to and where in the nematode intestine these cells are located.

Finally, it would be important to further investigate the structure of *mad* fimbriae. In order to better visualize *mad* fimbriae, immunogold electron microscopy of wild-type and mutant bacteria could be done. This could help to better determine structural differences between wild-type and mutant *mad* fimbriae, such as differences in length, number, or distribution on the bacterial surface. In the case where *mad* fimbriae are completely absent from mutant bacteria, this could also be detected by immunogold electron microscopy. For a more detailed look at the molecular structure of *mad* fimbriae, crystallography and X-ray diffraction could shed light on the exact structure and receptor binding sites. Since wild-type and mutant bacteria did not appear different under the TEM, qRT-PCR could be done to detect if other fimbrial genes are upregulated in *mad* mutants. In addition, flow cell experiments could answer the question if *mad* fimbriae promote adherence under flow.

APPENDICES

RNA ISOLATION AND RT-PCR OF *mad* GENE CLUSTER OPERON

Two results presented in Chapter 2 suggest that *P. luminescens* gene cluster VI might be an operon: 1) the *mad* gene cluster is regulated by an inverted repeat switch, and 2) no matter what *mad* gene the transposon inserted into, TRN *mad* mutants did not colonize *H. bacteriophora*. If the *mad* gene cluster is an operon, genes should be transcribed continuously and expression should be detected by RT-PCR. For this purpose, RNA was isolated from bacteria grown in Grace's Insect medium liquid cultures and reverse transcribed as described before. Primers used were as follows:

	forward	reverse
madR/madA	5'-acagccagtaatgctgaacg-3'	5'-tcacaatcgaaccggtaaca-3'
madA/madB	5'-acggttgctatgatcgttcc-3'	5'-atcaacggtgctggtgattt-3'
madB/madC	5'-gccagagctggcatacaaag-3'	5'-cgtgcaatttctcagcgtaa-3'
madC/madD	5'-ttcgtgataaaggggcagat-3'	5'-ccaggaacagcagaaccatt-3'
madD/madE	5'-tgttccggttgaatttgatg-3'	5'-aaagctggacccattcacac-3'
madE/madF	5'-gaaattggagggagcaatga-3'	5'-acggtaaactccgatgaacg-3'
madF/madG	5'-cctatggccgactgacattt-3'	5'-gactccgttgctggatgatt-3'
madG/madH	5'-gttgcagactttgcaggtca-3'	5'-gctccatcccgtcagaaata-3'
madH/madI	5'-agtgcgaggtggcatttaac-3'	5'-taaggtcgctttacccgttg-3'
madI/madJ	5'-cccaagctttctggtgagtt-3'	5'-tctgtcactcggaacacctg-3'

These primers were designed to amplify from the end of one gene to the start of the next gene to detect co-transcription of *mad* genes. Controls included DNaseI treated but not reverse transcriped RNA and genomic DNA. PCR products were analyzed for appropriate fragment size on a 1% agarose gel.

Results

In *P.* luminescens TT01 Tn7GFP, *madA* through *madF* were co-transcribed, with a gap in transcription from *madG* through *madI* (Table 1). In TRN26-25, there were gaps in transcription between *madB* and *madC*, *madD* through *madG*, and *madI* and *madJ*

(Table 1). In TT01 Tn7GFP, *madR* and *madA* were not co-transcribed, but they were in TRN26-25 (Table 1).

Table 1: Expression of *mad* genes as measured by RT-PCR.

Primer	TT01 Tn7GFP	TRN26-25	Putative protein
madR/madA	-	+	fimA
madA/madB	+	+	chaperone
madB/madC	+	-	fimA
madC/madD	+	+	fimA
madD/madE	+	-	adhesin
madE/madF	+	-	chaperone
madF/madG	-	-	chaperone
madG/madH	-	+	usher
madH/madI	-	-	secreted protein
madI/madJ	+	-	unknown

Discussion

It was not confirmed by this test that gene cluster VI in *P. luminescens* is an operon. The results were too inconclusive and the experiment needs to be repeated to optimize PCR conditions. However, it makes sense that *madR* and *madA* are not co-transcribed, otherwise it would defeat the purpose of the inverted repeat switch that was found upstream of *madA*. Perhaps, the RNA was not isolated during a time when all the genes are expressed, or the RNA was degraded before reverse transcription was completed. Also, secondary promoters could be distributed throughout the gene cluster. The fact that about half of the genes were expressed and half weren't suggests that the operon might have been in the beginning stage of being fully activated. Other problems could include pipetting errors during PCR setup, with some of the PCR tubes not receiving any cDNA or not enough. Also, either the reverse transcriptase or primers used could have been faulty and might have not worked. This could be tested by ordering new enzyme and primers.

GENE KNOCKOUT BY PCR STRAND OVERLAPPING EXTENSION

In order to reconstruct the TRN mutant phenotype in *P. luminescens* TT01, gene knockouts were attempted by using the PCR SOEing method adapted from Metcalf *et al.* 1996. Oligonucleotide primers for the flanking regions upstream and downstream of *madA* and *madH* were designed to amplify a ~500 bp fragment including at least the first and last six to eight codons. The oligonucleotide sequences were as follows:

madA Afor 5'-gtaccggccgtgctgaacgttttcaaggagt-3'

madA Arev 5'-ataggagaaggtgatgggggccgccacgacgcttatctttaa-3'

madA Bfor 5'-ttaaagataagcgtcgtggcggcccccatcaccttctcctat-3'

madA Brev 5'-tcgacggccgagggtccaaacgaaataaagg-3'

madH Afor 5'-gtaccggccgagcgcgtcagtaaaagaggat-3'

madH Arev 5'-attccagcgtttatctgccttaaataccagcatcacccgaca-3'

madH Bfor 5'-tgtcgggtgatgctggtatttaaggcagataaacgctggaat-3'

madH Brev 5'-tcgacggccgtaaggtcgct*ttacccgttgc-3'*

Afor and Brev primers also included an *EagI* linker. Half of Arev and Bfor were complimentary to each other to create a linking region. First, both fragments were amplified in separate PCR reactions. Fragment sizes were checked by agarose gel electrophoresis. Correct fragments were treated with ExoSap and diluted 1:500 with sterile ddH_2O. The diluted fragments were combined and amplified by PCR again using Afor and Brev primers to fuse the two fragments into one. Fragment sizes were checked again by agarose gel electrophoresis. Correct fragments were ligated into pCRII dual promoter cloning vector (Invitrogen).

The ligated vector plus insert was transformed into DH5α chemically competent cells (Invitrogen) and plated on MacConkey Agar containing 100 μg/mL ampicillin. DH5α cells contain a *lacZ* gene that allows for red-white screening on MacConkey Agar. The insert disrupts the *lacZ* gene on the vector and the cells are no longer able to utilize the lactose in the MacConkey Agar, resulting in colonies to be white. When colonies appeared, only white colonies were used for further screening, since they are likely to contain the vector plus insert. Transformants were screened for the correct insert by colony PCR using Afor and Brev primers. Plasmids of correct transformants were extracted and digested with *EagI* to release the insert. The insert was gel purified, ligated into vector pWM91 (digested with *EagI* and treated with Shrimp Alkaline Phosphatase), and electroporated into TransforMax EC 100 D *pir*-116 electrocompetent cells (EPICENTRE Biotechnologies). Cells were recovered in SOC broth and plated on MacConkey Agar Amp.

Transformants were screened for the correct insert by colony PCR using Afor and Brev primers followed by agarose gel electrophoresis. Plasmids of correct transformants were extracted, digested with *EagI*, and separated on a 1% agarose gel to check fragment sizes. Correct plasmids were electroporated into *E. coli* BW29427 cells. The cells were recovered in SOC broth and plated on LB DAP Amp. Transformants were screened for the correct insert by colony PCR using Afor and Brev primers followed by gel electrophoresis. Cells containing the correct insert were mated with TT01 Tn7GFP for 7 hours, plated on PP3S Amp in different volumes (25 μL, 50 μL, 100 μL, and 100 μL each of 10^4 and 10^5 dilutions), and incubated for two days. Ampicillin resistant colonies were further plated on PP3S Amp Suc 7.5% and PP3S Suc 7.5%. Colonies that were

ampicillin resistant and sucrose sensitive were tested for gene deletion by colony PCR using primers designed to amplify a region within the deleted gene followed by agarose gel electrophoresis. Oligonucleotide sequences were: madA del confirm for 5'-tgttaccggttcgattgtga-3', madA del confirm rev 5'-ggtttccaggagcatctgtg-3', madH del confirm for 5'-ggtagtgtggcaggtcaggt-3', and madH del confirm rev 5'-ccaggcagaccgtaaatcat-3'.

Results

Two of the genes in gene cluster VI where chosen for deletion based on their importance: 1) *madA* - the major structural subunit of fimbriae, and 2) *madH* - the outer membrane usher. After successful cloning of each gene deletion into *E. coli* BW29427, three independent matings of *P. luminescens* TT01 Tn7GFP with *E. coli* BW29427Δ*madA* or *E. coli* BW29427Δ*madH* were performed. During the mating, by means of a 2-step gene replacement, the targeted genes should be deleted. Colony PCR of the offspring revealed four possible TT01 Tn7GFPΔ*madH* knockouts and none for TT01 Tn7GFPΔ*madA*. However, when the possible TT01 Tn7GFPΔ*madH* knockouts were tested in *H. bacteriophora*, all nematodes were fully colonized by bacteria, indicating that the gene was not deleted.

Discussion

Although, *P. luminescens* gene knockouts in this study were not confirmed, it would still be important to continue efforts to reconstruct the mutations found in the NC1 Tn7GFP mutants in TT01 Tn7GFP. At this point, pWM91Δ*madA* and pWM91Δ*madH* were cloned into *E. coli* BW29427, and presence of the correct fragment was confirmed by PCR. The step that proved to be problematic was the mating of TT01 Tn7GFP with

BW29427Δ*madA* and BW29427Δ*madH*. Some of the problems that occurred during mating could probably be solved by optimizing the mating process. For example, mating time could be increased from 7 hours to an overnight (or 12 hour) mating. Also, ampicillin didn't seem to be very effective at keeping down background growth, which made it difficult to screen offspring by colony PCR after matings. Due to poor Ap selection, plasmid integration was not confirmed, and it would be prudent to move the deletion construct to a plasmid with a tighter antibiotic selection, like a Tet plasmid.

Reference

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BIOFILM FORMATION ON POLYSTYRENE PLATES

In order to test if *mad* fimbriae were major fimbriae, *P. luminescens* and *P. temperata* wild-type and TRN mutants were tested for biofilm formation on polystyrene plates (assay adapted from O'Toole and Kolter, 1998). If *mad* fimbriae are not a major type of fimbriae, TRN *mad* mutants should still be able to form biofilms on an abiotic surface.

Overnight bacterial cultures were diluted to an OD_{600} of 0.6, and 10 µL of each were used to inoculate a 96-well microtiter plate containing 100 µL Grace's Insect media per well. Rows with media blanks were also included. After reading the initial OD_{600}, the microtiter plates were parafilmed and incubated on wet paper towels for 48 hours at 28°C. After 48 hours, the OD_{600} was read again for the entire plate before removing the liquid. Each well received 150 µL of 0.1% crystal violet (in ethanol), and the plate was incubated at room temperature for 15 minutes. The stain was removed and the plates were rinsed with DI water three times by immersing. Each well received 200 µL of a 95% ethanol/5% acetic acid mixture to dissolve the stained biofilm. Absorbance was read at 590 nm with SpectraMax M5 plate reader (Molecular Devices Corporation, Sunnyvale, CA).

Results

Biofilm formation was not significantly different among the four wild-type strains TT01, TT01 Tn7GFP, NC1, and NC1 Tn7GFP (Figure 1). The majority of the fimbrial mutants tested were able to form biofilms on an abiotic surface at levels not significantly different from wild-type, with the exception of TRN359, TRN10-245, TRN10-329, TRN18-264, TRN19-124, and TRN22-68 (Figure 1).

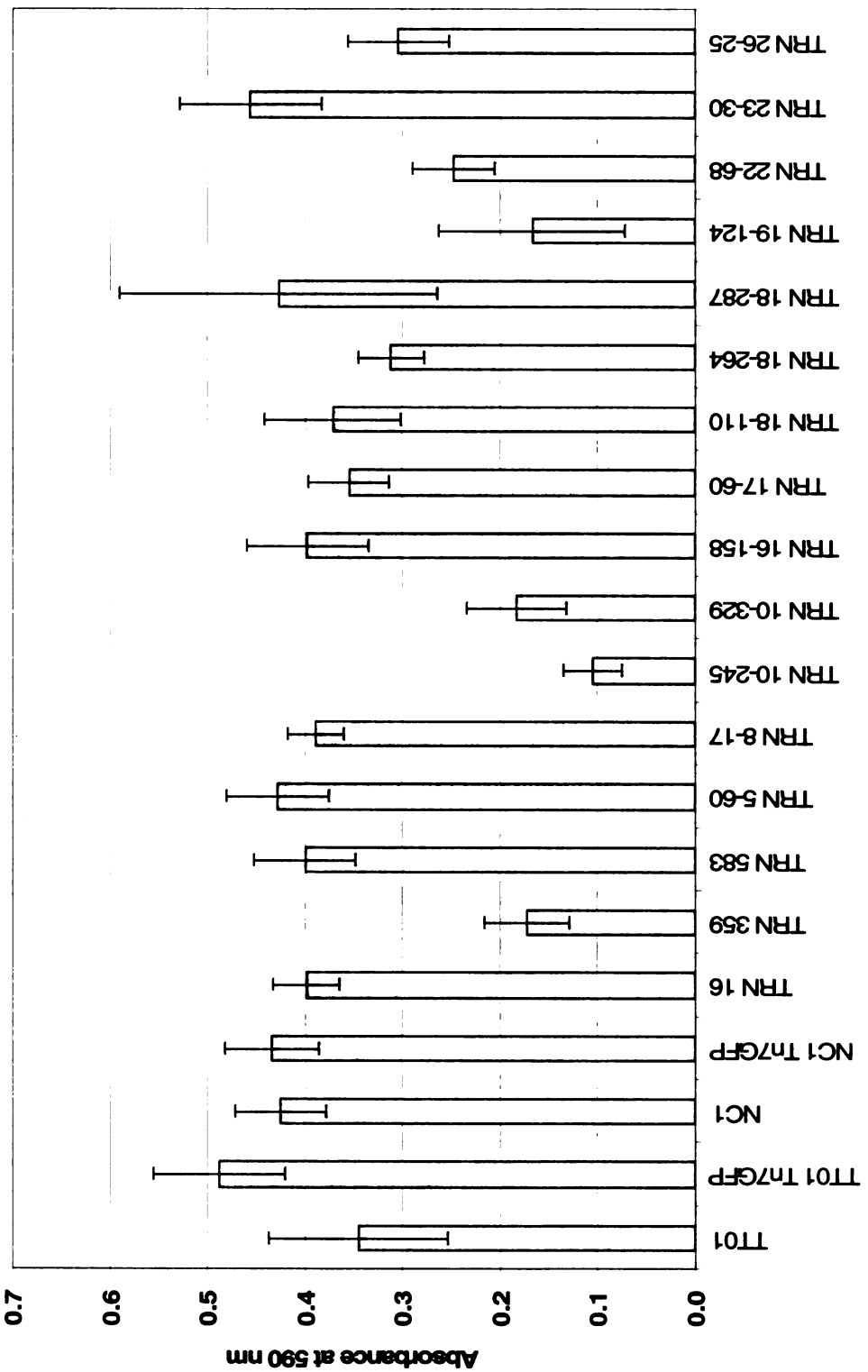

Figure 1: Biofilm formation of wildtype *P. luminescens* and TRN mutants on a 96-well plate. After 48 hours of incubation, the resulting biofilm was stained with 0.1% Crystal violet and dissolved in a 95% ethanol/5% acetic acid mixture. The absorbance was read at 590 nm.

Discussion

Since the majority of TRN mutants were able to form a biofilm on an abiotic surface, such as a microtiter plate, the fimbriae encoded by gene cluster VI must be specialized for attachment within the nematode intestine, since the remaining functional fimbrial gene clusters were sufficient for biofilm formation. It was a little surprising to see the variability in the wild-type strains, which should have displayed equal levels of biofilm formation. The Crystal violet should have been dissolved in water instead of ethanol. During the staining, the ethanol that was used to dissolve the Crystal violet probably dissolved some of the biofilm that had formed in the wells. When the plates were rinsed with DI water, a substantial amount of biofilm was washed away before it could be measured. Also, some of the TRN mutants showed low levels of biofilm formation, which could indicate an overall growth defect in these mutants due to the transposon insertion. Growth rates for *mad* mutants and wild-type strains could be measured by growing overnight cultures of wild-type strains and mutants and inoculating a 96-well plate with 10 μL of an overnight culture that was diluted to an OD_{600} of 0.6. Then, the 96-well plate could be placed in a SpectraMax M5 Spectrophotometer, which can be set to automatically take OD measurements at pre-set time intervals for a total of 16 or more hours. The resulting growth curves could be compared for any kind of growth defects. Also, different kinds of media, such as 2% PP3S and Grace's Insect media, could result in growth differences.

Reference

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