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## Molecular basis of *Corynebacterium diphtheriae* virulence and infection in the *Caenorhabditis elegans* model host

Melissa M. Broadway

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MOLECULAR BASIS OF *CORYNEBACTERIUM DIPHTHERIAE* VIRULENCE AND  
INFECTION IN THE *CEANORHABDITIS ELEGANS* MODEL HOST

by

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MOLECULAR BASIS OF *CORYNEBACTERIUM DIPHTHERIAE* VIRULENCE AND  
INFECTION IN THE *CAENORHABDITIS ELEGANS* MODEL HOST

A

THESIS

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of  
MASTER OF SCIENCE

by

Melissa M. Broadway, B.S.  
Houston, Texas

May 2010

## **Dedication**

I dedicate this thesis to my family and friends. Their continued support, understanding, and above all love throughout the years is appreciated more than they could ever know. The completion of this work would not have been possible without them.

## **Acknowledgements**

I would first like to thank Hung Ton-That, Ph.D., for his support and guidance throughout my thesis work. Thank you for not only teaching me technical skills, but also for your continued encouragement, without it this work would not have been a success.

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Thanks to the graduate school. Deans George Stancel, Thomas Goka, Jon Wiener and Victoria Knutson and the staff have been tremendously supportive and keep the environment here a wonderful place for students to learn and grow.

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My friends are exceptional. I thank you all for your optimism and encouragement. Jessica, thank you for always being on my side. Stephanie, thank you for always listening. Tom, thank you for having confidence in me, even when I do not have confidence in myself. Without you all, I would be lost.

I attribute my strength and character to my family. My parents unwavering love and faith in me is the motivating force behind my success. My siblings humor and inspiration cure a bad day. My grandparents comfort and assurance keep me cheerful through tough times. My cousins, aunts, and uncles are always there for additional support. Thank you all, I am grateful to have you in my life.

# **Molecular basis of *Corynebacterium diphtheriae* virulence and infection in the *Caenorhabditis elegans* model host**

Melissa M. Broadway, B.S.

Supervisory Professor: Hung Ton-That, Ph.D.

*Corynebacterium diphtheriae* is the causative agent of cutaneous and pharyngeal diphtheria in humans. While lethality is certainly caused by diphtheria toxin, corynebacterial colonization may primarily require proteinaceous fibers called pili, which mediate adherence to specific tissues. The type strain of *C. diphtheriae* possesses three distinct pilus structures, namely the SpaA, SpaD, and SpaH-type pili, which are encoded by three distinct pilus gene clusters. The pilus is assembled onto the bacterial peptidoglycan by a specific transpeptidase enzyme called sortase. Although the SpaA pili are shown to be specific for pharyngeal cells *in vitro*, little is known about functions of the three pili in bacterial pathogenesis. This is mainly due to lack of *in vivo* models of corynebacterial infection. As an alternative to mouse models as mice do not have functional receptors for diphtheria toxin, in this study I use *Caenorhabditis elegans* as a model host for *C. diphtheriae*. A simple *C. elegans* model would be beneficial in determining the specific role of each pilus-type and the literature suggests that *C. elegans* infection model can be used to study a variety of bacterial species giving insight into bacterial virulence and host-pathogen interactions. **My study examines the hypothesis that pili and toxin are major virulent determinants of *C. diphtheriae* in the *C. elegans* model host.** The specific aims and results are: **1. Investigate if a correlation between pilus**

**expression in corynebacterial clinical isolates and increased virulence in the *C. elegans* model exists.** In order to test this, I developed a *C. elegans* infection model to study *C. diphtheriae* virulence and characterized 46 clinical isolates of *C. diphtheriae* for the presence of toxin and the presence and expression of pili. After this characterization, representative isolates were examined in *C. elegans* killing. These data suggest that *C. diphtheriae* lacking toxin and any pilus gene cluster are attenuated in *C. elegans* killing. **2. Determine which *C. diphtheriae* pilus structure is a major virulence factor and which pilus component is important in infection.** The role of each pilus-type during infection has not been investigated. In order to test this, I used a large battery of corynebacterial mutants that express or lack individual pilus components in the *C. elegans* model to determine which pilus component is required for nematode killing. From this study I have shown *C. elegans* infection with *C. diphtheriae* does not require a specific pilus component.

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## **Chapter 1: Introduction**

## ***Corynebacterium diphtheriae***

*Corynebacterium diphtheriae*, an aerobic, nonmotile, club-shaped, Gram-positive bacillus, is the causative agent of cutaneous and pharyngeal diphtheria in humans (19). This disease is characterized by the formation of a mucous pseudomembrane at the site of infection either in the nasopharyngeal region or on skin lesions. The symptoms of diphtheria range greatly from pharyngitis, fever and swelling of the neck or area surrounding the lesion to potentially fatal airway obstruction and organ failure (19). The disease is a result of the combined effects of bacterial growth, toxin production, necrosis of underlying tissue, and the host immune response. Toxigenic strains of *C. diphtheriae* harbor corynebacteriophage, which carries the *tox* gene that encodes diphtheria toxin (DT). DT inhibits protein synthesis which promotes cell death and is able to enter the circulatory system and act on peripheral neurons and the myocardium (5, 10). Nontoxigenic strains of *C. diphtheria* can be stably infected by a corynebacteriophage that carries the *tox* gene, therefore, making them toxigenic. *C. diphtheriae* is classified into four biotypes according to colony morphology and corynebacteriophage sensitivity. These biotypes include: *gravis*, *intermedius*, *belfanti*, and *mitis*, the most severe clinical form of diphtheria is associated with *gravis* (28).

Control of diphtheria depends on immunization with the diphtheria toxoid vaccine. The toxoid is prepared by incubating DT at 37°C with formaldehyde, which renders the toxin inactive but still antigenic. The diphtheria toxoid vaccine has been shown to be 97% effective and is given as a component in the DPT vaccine (diphtheria, pertussis, tetanus) (19). While DT is the major virulence factor

expressed by *C. diphtheriae*, recently there has been a worldwide emergence of non-toxigenic *C. diphtheriae* strains causing systemic infections (26). Although vaccination prevents toxin-mediated diphtheria, non-toxigenic *C. diphtheriae* may express other factors capable of causing different diseases such as pharyngitis and endocarditis. (26). One aspect that has yet to be investigated is the ability of *C. diphtheriae* to colonize and successfully compete in the nasopharyngeal niche (19). Of the many possible *C. diphtheriae* colonization factors, pili may play an important role in host colonization. My study focuses on *C. diphtheriae* pili as potential virulence factors.

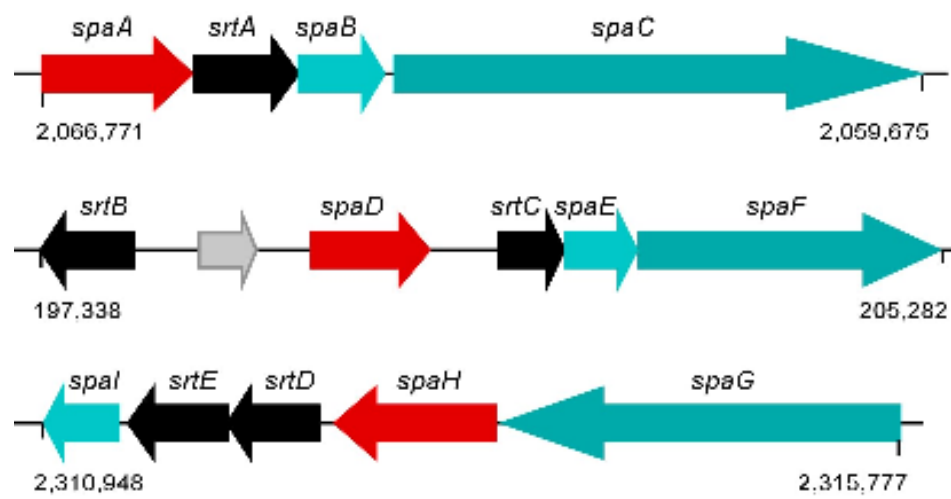
### ***Corynebacterium diphtheriae* express three distinct pilus structures**

Pili were first described in Gram-negative bacteria as filamentous structures extending from bacterial surfaces in the 1950s (32). It was twenty years later, in the 1970s, that Gram-positive pili were first observed by electron microscopy in *C. diphtheriae* (35). However, not until 2003 was the mechanism of pilus assembly in Gram-positive bacteria described using *C. diphtheriae* as an experimental system (33). Initial studies in *Staphylococcus aureus*, a Gram-positive pathogen, discovered the sortase enzyme, which catalyzes cell wall anchoring of surface proteins containing the cell wall sorting signal with the LPXTG motif (16). Bioinformatic analysis investigating *C. diphtheriae* for the presence of sortase homologs revealed three distinct pilus gene clusters (namely *spaA*, *spaD*, and *spaH*) (Figure 1); each encodes three surface proteins with the LPXTG motif and

**Figure 1: Representation of pilus gene clusters in *C. diphtheriae* NCTC13129.**

Pilus-specific sortase gene(s) are black, major pilin subunit genes are red and minor pilin subunit genes are aqua. Genes of unknown function are colored in gray.

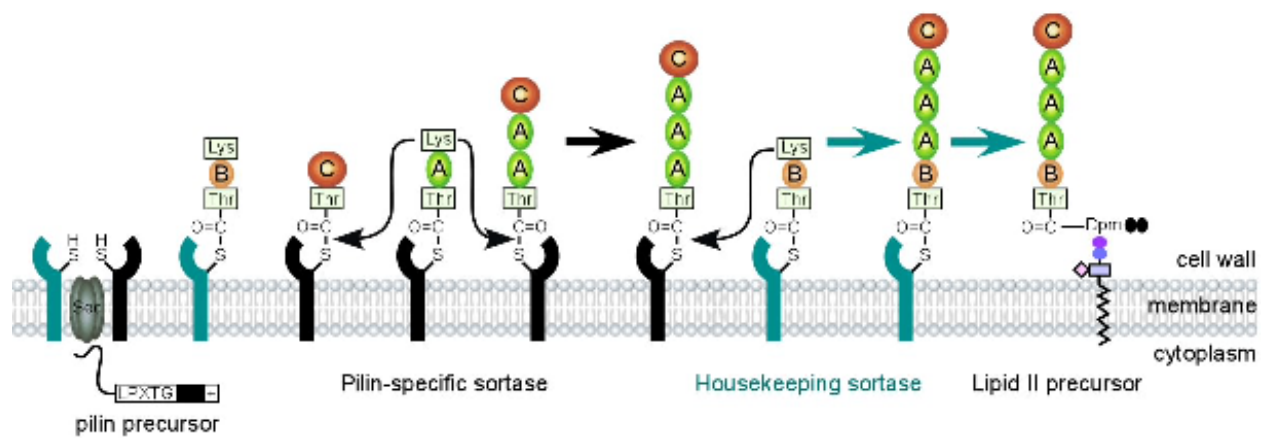
Numbers below clusters indicate the genomic location of pilus gene clusters (13).



one or two sortases (33). In addition to five pilus-specific sortases (SrtA-E), *C. diphtheriae* possess a sixth housekeeping sortase located elsewhere in the chromosome (SrtF). It has been shown that *C. diphtheriae* assembles SpaA-, SpaD-, and SpaH-type pili (spa for sortase-mediated pilus assembly) and the assembly of each pilus type is sortase-mediated (33). The sortase encoded within each pilus gene cluster specifically catalyzes the covalent cross linking of individual pilin monomers of that pilus type (13). Sortases are conserved in all Gram-positive bacterial genomes sequenced to date and many Gram-positive pathogens contain pili that are organized in a similar way as *C. diphtheriae* pili (13, 14). Therefore, investigations into corynebacterial pili will also shed light on pili in other Gram-positive bacteria.

All three *C. diphtheriae* pilus types are organized in a similar fashion. For example, the SpaA-type pili are composed of a major pilin subunit SpaA, which forms the pilus shaft; a minor pilin subunit SpaB, which decorates the shaft and is found at the base of the pilus; and SpaC which is a tip protein. All three pilin subunit proteins are synthesized as precursor proteins with an N-terminal signal peptide and a C-terminal cell wall sorting signal. The signal peptide is responsible for targeting precursor proteins for secretion via the secretion pathway machinery. Using SpaA-type pili as the prototype, a mechanism of pilus assembly has been proposed (Figure 2) (11). Once at the exoplasm, these precursor proteins are processed by pilus-specific sortase SrtA, they then form acyl-enzyme intermediates with the substrates. SrtA catalyzes pilus polymerization via a nucleophilic attack by

**Figure 2: Diagram of the biphasic mode of *C. diphtheriae* pilus assembly.** At the exoplasm, precursor pilin proteins form acyl-enzyme intermediates with either the pilus-specific sortase (black) or the housekeeping sortase (teal). The pilus-specific sortase catalyzes pilus polymerization via a nucleophilic attack by the amino group of the lysine in the pilin motif. Anchoring occurs when the housekeeping sortase, which already has a monomer of SpaB, receives the pilus polymer from the pilus-specific sortase and covalently attaches the polymer to the cell wall by joining the cell wall sorting signal threonine to the amino group of the peptidoglycan cross bridge (11).



the amino group of the lysine in the pilin motif (13). This covalent tethering of adjacent pilin subunits appears to have evolved in many Gram-positive pathogens, all of which encode sortases and pilin subunit genes with sorting signals and pilin motifs (13).

Recent studies have shown that corynebacterial pili may mediate adhesion to host tissues. Using *in vitro* tissue cultures, Mandlik et al showed that the deletion of the *spaA* gene abolishes pilus assembly, but does not abolish adherence to pharyngeal cells (12). Adherence is, however, greatly diminished when either minor pilin subunit, SpaB or SpaC, is absent. Studies have shown these minor pilins are also anchored to the cell surface in the absence or presence of a pilus shaft by the LPXTG motif. Thus, these minor pilin subunits may also be cell wall anchored in addition to their incorporation into pilus structures, and this could facilitate tight binding to host cells during bacterial infection (12).

In addition to the function corynebacterial pili play in pathogenesis, the literature has shown that a variety of Gram-positive pathogens possess pili that are virulence factors. Initial studies investigating the role of Gram-positive pili in pathogenesis were performed in *Actinomyces naeslundii* (*A. naeslundii*), an oral pathogen. These studies revealed that *A. naeslundii* possess pili that are involved in adhesion to salivary proteins and aide in biofilm formation in the oral cavity (32). Further evidence of the role of Gram-positive pili in pathogenesis comes from recent studies of *Streptococcus pneumoniae* (*S. pneumoniae*). These studies revealed that piliated strains of *S. pneumoniae* adhere better to lung epithelial cells than strains lacking pili. Mice infected with piliated *S. pneumoniae* also exhibited a greater

immune response compared to mice infected with non-piliated *S. pneumoniae* (32). Likewise, investigations comparing rats infected with piliated *E. faecalis* to non-piliated mutants, over 50% of the mutant strains were cleared from rat vegetation and kidneys while the parental infection persists (11). Additional confirmation that pili are key virulence factors in Gram-positive pathogens came from a study using nonpathogenic Gram-positive *Lactococcus lactis* (*L. lactis*) expressing a major pilin subunit from the Gram-positive pathogen *Streptococcus agalactiae* (*S. agalactiae*). The mutant *L. lactis* expressing the major pilin subunit was lethal in a mouse model of infection compared to non-lethal parental lactococci (11). This experiment demonstrates that the major pilin subunit of *S. agalactiae* is a unique factor involved in colonization and survival in the mouse. While all of these functions are known about Gram-positive pili, little is known about the role of each corynebacterial pilus type during infection. My study aims to elucidate the role of *C. diphtheriae* pili in pathogenesis.

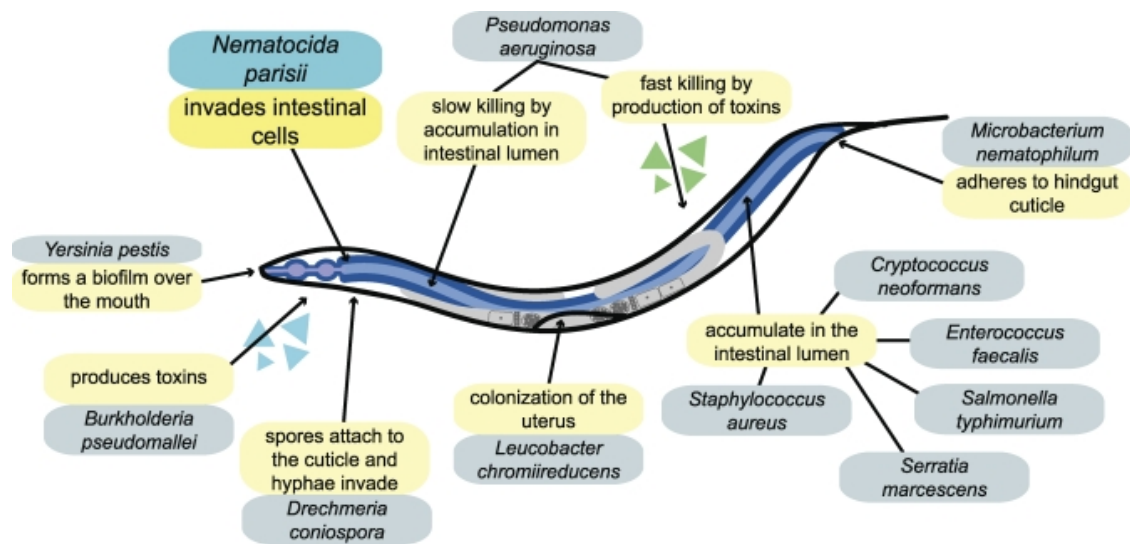
### ***Caenorhabditis elegans* as a model host for bacterial pathogenesis**

The nematode *Caenorhabditis elegans* (*C. elegans*) is a free-living, non-parasitic, soil-dweller commonly found around the world (25). The nematode is about one millimeter in length and transparent, making laboratory manipulation and observation easy. It feeds on bacteria, such as non-pathogenic *Escherichia coli* (*E. coli*), and can be stored on small agar plates (25). Microbes taken into the pharynx are crushed by a grinder, and pumped to the intestine. Nutrients are absorbed from the intestinal lumen by epithelial cells and unabsorbed material is excreted through the anus (6).

*C. elegans* has two sexes, hermaphrodites and males. In nature, hermaphrodites are the most common and self-fertilization can produce approximately three hundred offspring (34). The development of *C. elegans* consists of four larval stages (L1-4) before reaching the adult stage. The life cycle is conveniently short, only taking approximately three days to grow from egg to adult at 25°C. Under laboratory conditions *C. elegans* has a lifespan of approximately two weeks (9).

*C. elegans* has recently been shown to be an efficient and effective animal model for studies of bacterial pathogenesis (31). A variety of bacterial pathogens have been found to infect and kill *C. elegans*, allowing for the study of many different types of virulence factors (Figure 3) (22, 30, 31). Many of the virulence mechanisms used by pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*, in the *C. elegans* model have also been shown to be important for disease in mammalian hosts (31). The innate immune system in the nematode has been well documented and shown to be closely related to human innate immunity (31). The advantage of using the *C. elegans* model system to study bacterial pathogens is that both the host and the pathogen are able to be genetically manipulated, allowing for the identification of bacterial virulence factors and host immune defenses (31). Many *C. elegans*-based pathogenesis systems have been elucidated over the past few years and *C. elegans* continues to be a popular host for studying bacterial virulence (8, 31). All of these reasons have led to the investigation of *C. elegans* as a model host for *C. diphtheriae* infection in this study.

**Figure 3: *C. elegans* host-pathogen interactions with various microbial species.** Diagram of *C.elegans* demonstrating some of the pathogens of the nematode (highlighted in blue) and the mode of infection of each pathogen (highlighted in yellow) (8).



A novel *C. elegans* pathogen first observed by the Hodgkin laboratory has been shown to infect the nematode and cause a distinctive swollen tail. The bacteria adhere to the rectal and post-anal cuticle of the infected nematodes, and cause swelling of the underlying hypodermal tissue. This tail swelling is accompanied by constipation and slowed growth in the worms, but the infection is otherwise non-lethal (7). The phenotype is called Dar (deformed anal region), and the Hodgkin group found that the infectious agent responsible for this distinctive swollen tail is a novel species of coryneform bacterium called *Microbacterium nematophilum* (*M. nematophilum*). They thought initially that the Dar phenotype was a genetic trait, but they examined the worms by light microscopy and found a small patch of bacteria sticking to the cuticle immediately behind the anus (7). Upon further investigation of this host-pathogen interaction, it was observed that an extracellular signal-regulated kinase mitogen-activated protein (ERK-MAP) kinase cascade was acting as an innate immune response mechanism during infection (21). This ERK-MAP kinase cascade is required for tail swelling. When mutant *C. elegans* defective in producing multiple components of the ERK-MAP kinase cascade are infected with *M. nematophilum*, no tail swelling occurs. These mutants also had an increase in susceptibility to constipation. Thus, the authors concluded that the tail swelling that occurs in *C. elegans* infected with *M. nematophilum* helps control the infection in the nematode (21). In this study I observed *C. elegans* fed on *C. diphtheriae* exhibit the Dar phenotype. This phenomenon will be discussed in more depth in results section (Chapter 3).

This work explores the use of *C. elegans* as a model host to investigate the mechanism of corynebacterial infection. **My study examines the hypothesis that pili and toxin are major virulent determinants of this deadly pathogen in the *C. elegans* model host. The first specific aim is to investigate corynebacterial clinical isolates to determine if a correlation between pilus expression and increased virulence in the *C. elegans* model host exists.** In order to do this I characterized 46 clinical isolates of *C. diphtheriae* for the presence of toxin and the presence and expression of pili. Based on this characterization, representative isolates were observed in *C. elegans* killing. These data suggest that *C. diphtheriae* lacking toxin and any pilus gene cluster are attenuated in *C. elegans* killing. **The second specific aim is to determine which *C. diphtheriae* pilus structure is a major virulence factor and which pilus component is important in infection in the *C. elegans* model host.** In order to test this, I used a large array of corynebacterial mutants that lack individual pilus components. These mutants were examined in the *C. elegans* model to determine which pilus component is required for pathogenesis in the nematode. From these data I have shown that a specific *C. diphtheriae* pilus component is not required for *C. elegans* infection. While undertaking this process, it was observed that nematodes infected with *C. diphtheriae* exhibit the Dar phenotype described above. The results introduce a novel animal model for *C. diphtheriae* pathogenesis and provide insight into the distribution of different pilus types in *C. diphtheriae* clinical isolates.

## **Chapter 2: Materials and Methods**

## **Bacterial strains and media**

*Corynebacterium diphtheriae* NCTC13129, parental strain, was obtained from the American Type Culture Collection (ATCC), and the 46 *C. diphtheriae* clinical isolate strains were obtained from the Centers for Disease Control and Prevention and collaborators. The clinical specimens were collected from patients with diphtheria between 1997 and 2000. All *C. diphtheriae* strains (Table 1) were grown on heart infusion broth (HIB) and heart infusion agar (HIA). *E. coli* strains were grown in Luria broth. Kanamycin was added 50 µg ml<sup>-1</sup> as needed.

## **Chromosomal DNA extraction and standard polymerase chain reaction (PCR)**

Chromosomal DNA was extracted from all of the clinical isolates using the Promega Wizard® Genomic DNA Purification Kit according to the manufacture's protocol. Once chromosomal DNA was extracted, using specific primers to target genes encoding the major pilin subunits, DT, and the 16S ribosomal subunit, PCR analysis was performed as previously described (20) . Multiple primer pairs were designed from the genome sequence of the type strain (NCTC13129) for this study (Table 2) (1).

## **Extraction of *Corynebacterium diphtheriae* pili**

*C. diphtheriae* pili were extracted from the cell wall as previously described (33). Briefly, cells were scraped from trypticase soy agar supplemented with 5% sheep blood after overnight growth at 37°C and washed in SMM buffer

**Table 1: Genotypic and phenotypic characterization of *C. diphtheriae* clinical isolates.** Characterization of 46 clinical isolates of *C. diphtheriae* for the presence of *tox* gene and the presence and expression of major pilin subunits. Control strains (C.S.) used in this analysis include the type strain, an isogenic mutant lacking all major pilin subunits ( $\Delta spaADH$ ), and two strains known to be lacking (C7-) or expressing (C7 $\beta$ ) *tox*. Based on this characterization the clinical isolates that contain no *tox* and no major pilin subunits form group 1. Other isolates that contain no *tox*, but have one major pilin subunit form group 2. Group 3 contains no *tox*, but has two major pilin subunits. Group 4 contains *tox* (+) but no major pilin subunits. The fifth and sixth groups contain *tox* and one or two types of major pilin subunits. The seventh group contains all major pilin subunits, as well as, *tox*.

c.s.	Origin <sup>A</sup>	Strain	Biotype <sup>B</sup>	tox analysis			spaA analysis		spaD analysis		spaH analysis	
				CDC	PCR <sup>C</sup>	PCR	PCR	Western	PCR	Western	PCR	Western
1	ATCC	NCTC13129	G	+	+	+	+	+	+	+	+	+
	NCTC13129	$\Delta$ spaADH	G	+	+	+	-	-	-	-	-	-
	US, California	C7 (-)	M	-	-	-	-	-	-	-	-	-
	US, California	C7 (B)	M	+	+	+	-	-	-	-	-	-
	Canada	CD25/C65	M	-	-	-	-	-	-	-	-	-
	Canada	CD26/C64	B	-	-	-	-	-	-	-	-	-
	Canada	CD29/C78	B	-	-	-	-	-	-	-	-	-
	Kazakhstan	CD30/C50	B	-	-	-	-	-	-	-	-	-
	US, Ohio	CD310	B	-	-	-	-	-	-	-	-	-
	US, Maine	CD317	B	-	-	-	-	-	-	-	-	-
2	US, Colorado	CD376	B	-	-	-	-	-	-	-	-	-
	Russia	G4193	G	-	-	-	-	-	-	-	-	-
	Kazakhstan	CD31/C52	M	-	-	-	+	+	-	-	-	-
	Russia	CD44/760	M	-	-	-	+	+	-	-	-	-
	US, Maine	CD318	G	-	-	-	+	+	-	-	-	-
	US, South Dakota	CD53/PR26	G	-	-	-	+	+	-	-	+	+
	US, South Dakota	CD54/PR120	G	-	-	-	+	+	-	-	+	+
	US, New York	CD364	M	-	-	-	+	+	-	-	+	+
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<sup>A</sup>All strains, except CD55/A12, were isolated from humans (18) .

<sup>B</sup> M, *mitis*; B, *belfanti*; G, *gravis*; I, *intermedius*.

<sup>C</sup> Previously published data confirming the toxigenicity of all strains by standard PCR (18).

Origin <sup>A</sup>	Strain	Biotype <sup>B</sup>	tox analysis		spaA analysis		spaD analysis		spaH analysis	
			CDC	PCR <sup>C</sup>	PCR	Western	PCR	Western	PCR	Western
4	Russia	CD082	-	+	+	-	-	-	-	-
	Russia	CD081	+	+	-	-	-	-	-	-
	Russia	CD32/C496	+	+	-	-	-	-	-	-
	Russia	CD39/718	+	+	-	-	-	-	-	-
	Russia	CD40/713	+	+	-	-	-	-	-	-
	Russia	CD42/724	+	+	-	-	-	-	-	-
	US, South Dakota	CD49/C5276	+	+	-	-	-	-	-	-
	US, South Dakota	CD50/PR110	+	+	-	-	-	-	-	-
	US, South Dakota	CD52/G4219	+	+	-	-	-	-	-	-
	US, California	CD198	-	+	-	-	-	-	-	-
	US, Rhode Island	CD023	-	+	-	-	-	-	-	-
	Ukraine	1751	+	+	+	+	+	+	+	+
	Russia	CD080	-	+	+	+	+	+	+	+
	Russia	CD36/G4212	+	+	+	+	+	+	+	+
5	Russia	CD37/1899	+	+	+	+	+	+	+	+
	Russia	CD38/711	+	+	+	+	+	+	+	+
	Russia	CD43/G4182	+	+	+	+	+	+	+	+
	Russia	CD45/1709	+	+	+	+	+	+	+	+
	Russia	CD46/750	+	+	+	+	+	+	+	+
	US, South Dakota	CD48/PR75	+	+	+	+	+	+	+	+
	US, South Dakota	CD51/G4217	+	+	+	+	+	+	+	+
	US, VA horse	CD55/A12	+	+	+	+	+	+	+	+
	Canada	CD028	+	+	+	+	+	+	+	+
	Russia	CD35/722	+	+	+	+	+	+	+	+
	US, South Dakota	CD83/E8277	+	+	+	+	+	+	+	+
	Russia	CD41/765	+	+	+	+	+	+	+	+
	Russia	1716	+	+	+	+	+	+	+	+
	Russia	1718	+	+	+	+	+	+	+	+
7	Russia	1737	+	+	+	+	+	+	+	+
	Ukraine	1897	+	+	+	+	+	+	+	+
	Russia	CD33/G4174	+	+	+	+	+	+	+	+
	Russia	CD34/749	+	+	+	+	+	+	+	+
	Russia	CD34/749	+	+	+	+	+	+	+	+

<sup>A</sup> All strains, except CD55/A12, were isolated from humans (18) .

<sup>B</sup> M, *mitis*; B, *belfanti*; G, *gravis*; I, *intermedius*.

<sup>C</sup> Previously published data confirming the toxigenicity of all strains by standard PCR (18).

**Table 2: PCR primers specific for the three major pilin subunits, diphtheria toxin, and the 16S ribosomal subunit of *C. diphtheriae*.** Three primer sets were designed for each major pilin subunit, *spaA*, *spaD*, and *spaH*. The 16S ribosomal subunit was analyzed to confirm all isolates are *C. diphtheriae*. The *tox* gene was analyzed to confirm toxigenicity. Primer3 Input 0.4.0 (27) was used to select appropriate primer sequences.

Primer sets	Primer sequence (5'-3')	Position (range in NCTC13129)
<i>spaA</i> <sup>1</sup> Forward	GTGGGGGAGAACGTGAAGTA	673-693
<i>spaA</i> <sup>1</sup> Reverse	TCTTGGCCGGTAACAAACTC	1226-1246
<i>spaA</i> <sup>2</sup> Forward	ACGTCACACTTGCCTCAGTG	62-81
<i>spaA</i> <sup>2</sup> Reverse	CGGTGAAACCCTCGAACTTA	715-735
<i>spaA</i> <sup>3</sup> Forward	CCGTACTCAGCGTGCAACTA	839-859
<i>spaA</i> <sup>3</sup> Reverse	CCTGTGAGTGGCAGTTCAAA	1484-1504
<i>spaD</i> <sup>1</sup> Forward	GGCATCTACCTCGTTGAGGA	193-213
<i>spaD</i> <sup>1</sup> Reverse	TGCCGTCCTTCTTAACAACC	790-810
<i>spaD</i> <sup>2</sup> Forward	TGCTGGTGAGCCACTAAATG	12-32
<i>spaD</i> <sup>2</sup> Reverse	CCCGACTTGACCTTCTTCAG	614-634
<i>spaD</i> <sup>3</sup> Forward	TACCCGAAGAACCCGAAAC	313-333
<i>spaD</i> <sup>3</sup> Reverse	TTTGTACACCGTCCACAGTC	892-912
<i>spaH</i> <sup>1</sup> Forward	ACGGCAATATCGACTTCACC	140-160
<i>spaH</i> <sup>1</sup> Reverse	TCAGACCCGACGTTAAGACC	674-694
<i>spaH</i> <sup>2</sup> Forward	GAAGGTGGCTGAGGACAAAG	705-725
<i>spaH</i> <sup>2</sup> Reverse	TACTCCGGACCACAAGTTCC	1265-1285
<i>spaH</i> <sup>3</sup> Forward	GACCCGACCAAAGACGATAA	361-381
<i>spaH</i> <sup>3</sup> Reverse	CAGCAGCATTCTTCAGCTTG	910-930
<i>tox</i> Forward	AACGGCATTAGAGCATCCTG	717-737
<i>tox</i> Reverse	ATAGCTCTGCAACGCATCCT	1352-1372
16S Forward	CGGAATTACTGGGCGTAAAG	502-522
16S Reverse	CTCTCATGAGTCCCCACCAT	1103-1123

(0.5 M sucrose, 10 mM MgCl<sub>2</sub>, and 10 mM maleate, pH 6.8). Cells were then treated with muramidase (300 U ml<sup>-1</sup>) at 37°C for 4 hours to release cell wall anchored pili. Soluble pilus proteins were precipitated with 7.5% trichloroacetic acid. Samples were boiled in sample buffer containing sodium dodecyl sulfate (SDS), separated by SDS-polyacrylamide gel electrophoresis, subjected to immunoblotting with rabbit antisera (1:20,000 for α-SpaA and 1:5,000 for α-SpaD and α-SpaH) and detected with chemiluminescence. Polyclonal antibodies raised against recombinant pilins were obtained as previously described (4, 33). Polyclonal antibody raised against diphtheria toxin was purchased from Santa Cruz laboratories (1:200).

### **Generation of *Corynebacterium diphtheriae* deletion mutants**

Non-polar, in-frame *C. diphtheriae* deletion mutants were obtained via homologous recombination according to a published protocol (12, 33). Briefly, *E. coli* S17-1 (2, 29) carrying a gene deletion construct in pK19*mobsacB* was used for mating with appropriate *C. diphtheriae* strains. pK19*mobsacB* can be mated into *C. diphtheriae* and the conjugative plasmid carrying the homologous corynebacterial DNA sequence integrates into the *C. diphtheriae* chromosome via Campbell type insertion (33) Co-integrates resulting from this conjugation were identified on HIA plates supplemented with 25 µg ml<sup>-1</sup> nalidixic acid and 50 µg ml<sup>-1</sup> kanamycin. The deletion mutants were then selected by plating cointegrates on HIA plates containing 10% sucrose and 25 µg ml<sup>-1</sup> nalidixic acid. Gene deletions in these bacteria were confirmed by PCR and Western blotting.

### **Generation of fluorescently labeled *Corynebacterium diphtheriae***

Yellow Fluorescent Protein (YFP) was cloned into the integration vector pK-PIM (23). This plasmid exploits an integrase protein and *attP* site of a corynebacteriophage. This allows for integrase-dependent, site-specific recombination of the plasmid into the *C. diphtheriae* chromosome. A pK-PIM containing YFP was transformed into *E. coli* S17-1 and used for mating with appropriate *C. diphtheriae* strains. Co-integrates resulting from this conjugation were identified as described above.

### ***Caenorhabditis elegans* strains and maintenance**

The wild type *C. elegans* strain N2 was obtained from the *Caenorhabditis* Genetics Center, care of Danielle Garsin, Ph.D., University of Texas at Houston Medical School. The nematodes were grown and maintained as previously described (9) on nematode growth (NG) agar containing a bacterial lawn of OP50 *E. coli*.

### ***Caenorhabditis elegans* killing assays**

Killing assays were performed as previously described (3). *C. diphtheriae* was grown in brain heart infusion (BHI) broth overnight at 37°C and 15µl of overnight culture was spread onto BHI agar, supplemented with 25 µg ml<sup>-1</sup> nalidixic acid and 50µg ml<sup>-1</sup> 5-fluoro-2-deoxyuridine (FuDR), and incubated at 37°C to allow for a lawn of bacteria to grow. At the L4 young adult stage, nematodes were

transferred to plates containing *C. diphtheriae* strains then incubated at 25°C for the remainder of the experiment. Approximately 90 nematodes were used per *C. diphtheriae* strain and the assays were repeated two times. Every 24 hours the nematodes were counted and dead nematodes were removed for the killing assays. GraphPadPrism 5.0 was used to determine the Kaplan-Meier survival plots and the curves were compared using the log-rank test, which generates *P*-values testing the null hypothesis that the survival curves are identical. The *P*-values were used to determine the significance for each strain, where *P*-values less than 0.05 were considered significant. These data were fitted to Boltzmann sigmoidal curves, from which the LT<sub>50</sub> (lethal time 50, which describes the time in which half of the worms die) was assessed for each *C. diphtheriae* strain.

### **Fluorescence microscopy**

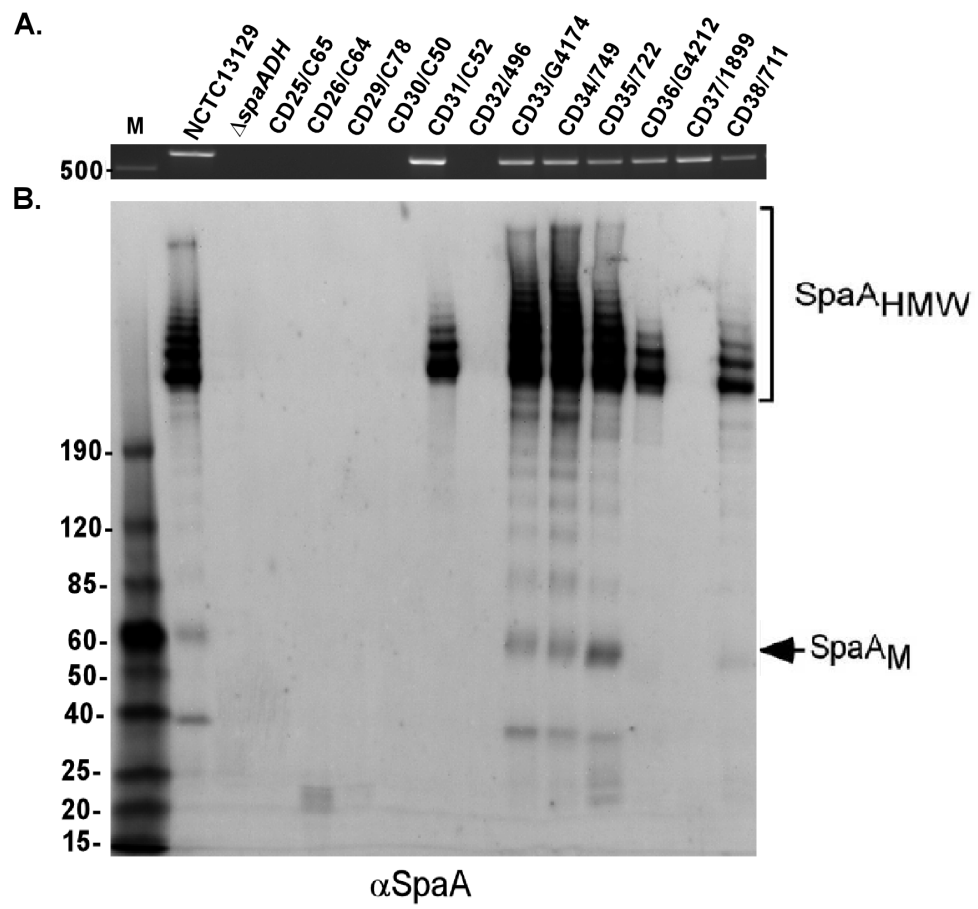
Nematodes were washed off of infection plates (BHI with a lawn of *C. diphtheriae*), anesthetized with 0.1% tetramisole HCl to induce paralysis, and then placed on 5% agarose pad microscope slides. Microscopy was performed on an Olympus inverted microscope under 60X magnification. All photographs represent what was typically seen upon examination and were taken at the same exposure and the levels of fluorescence intensity were manipulated identically. 50 worms were viewed for each condition. Each condition was repeated two times.

## **Chapter 3: Results**

## **Genotypic and phenotypic variation of pilus genes in *Corynebacterium diphtheriae* clinical isolates**

As mentioned previously, little is known about the three *C. diphtheriae* pili in bacterial pathogenesis. I therefore wanted to investigate *C. diphtheriae* clinical isolates to investigate if there was a correlation between pilus expression and virulence exists. Initially I sought to genotypically and phenotypically characterize 46 clinical isolates obtained from the Center for Disease Control and Prevention (Table 1). To begin genotypic characterization, chromosomal DNA was extracted from each isolate for PCR analysis. The toxin gene (*tox*) and the major pilin subunits of each pilus type (*spaA*, *spaD*, and *spaH*) were PCR amplified. As a control to ensure each isolate was *C. diphtheriae*, the 16S ribosomal subunit gene was also PCR-amplified from all isolates. Due to potential sequence divergence among the clinical isolates, multiple combinations of primers (Table 2) designed based on the type strain, were used for PCR amplification of each pilus type. The control strains (C.S) used in these experiments (Table1) include the type strain, an isogenic strain lacking all three major pilin subunits ( $\Delta spaADH$ ), as well as a DT-negative (C7-) and a DT-positive strain (C7 $\beta$ ). Figure 4A is a typical agarose gel depicting PCR results. PCR results are summarized in Table 1. In comparing the PCR results obtained from this study with those provided by the CDC for the *tox* gene, most isolates yielded consistent results. However, in four isolates that the CDC listed as lacking toxin, I was able to amplify the toxin gene. This is likely due to different primer combinations used in this study.

**Figure 4: Genotypic and phenotypic characterization of *C. diphtheriae* clinical isolates.** (A) Representative PCR amplification of the major pilin subunit *spaA*. Chromosomal DNA extraction was performed with each of the isolates and the DNA was then used for PCR amplification. (B) Pili were extracted by digesting from the cell wall with muramidase for 4 hours, and pilus proteins were precipitated and boiled in SDS with reducing agent. The high molecular weight (HWM) polymers seen upon immune-blotting with  $\alpha$ SpaA are indicative of pilus proteins, but monomeric (M) forms of the major pilin subunits can also be seen on these blots. Stain/isolate names are listed at the top. Size markers (M) are listed to the left and are in base pairs (A) and kilo Daltons (B).



To phenotypically characterize the clinical isolates, pilus expression was analyzed. Pili were extracted from the cell wall by digestion with muramidase; the proteins were precipitated, and fractionated by SDS-PAGE. After Ister blot analysis, two forms of pilus proteins are seen (Figure 4B). Monomeric forms of pilus proteins (SpaA<sub>M</sub>) can be seen, as well as high molecular weight polymers (SpaA<sub>HMW</sub>). The high molecular weight polymers are covalently linked, and are thus resistant to the reducing abilities of an SDS-PAGE gel (33).

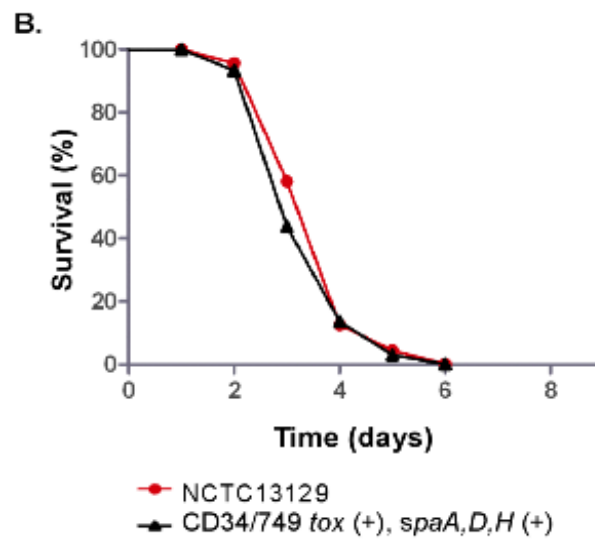
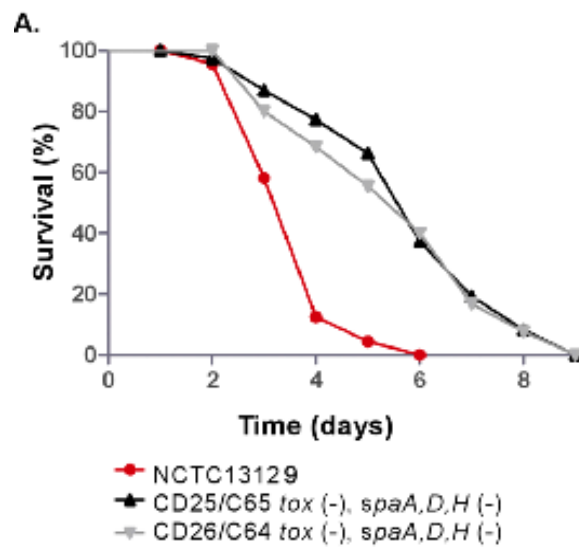
Based on the presence or absence of toxin and pilus genes (i.e. *tox*, *spaA*, *spaD*, and *spaH*), the 46 clinical isolates were classified into seven subgroups (Table 1). Group 1 contains isolates that have no *tox* and no major pilin subunit genes. Group 2 does not have *tox*, but has one major pilin subunit gene. Group 3 does not have *tox*, but has two major pilin subunit genes. Group 4 has the *tox* gene, but no major pilin subunit genes. Isolates in group 5 have *tox* and one major pilin subunit gene. Group 6 contains isolates that have *tox* and two major pilin subunit genes. Isolates in group 7 have a similar genotype as the type strain, i.e. having *tox* and all three major pilin subunit genes. There was a natural variation in the distribution of toxin and the three pilus gene clusters. Furthermore, the majority of isolates expressed at least one of the major pilin subunits, with SpaA expressed in all of these isolates. This indicates that Spa proteins are likely key mediators during adherence and infection; especially SpaA pili which have been shown to be major adhesion factors that bind to pharyngeal cells (12).

## ***Corynebacterium diphtheriae* clinical isolates lacking toxin and pili are attenuated in nematode killing**

While undertaking the characterization of these clinical isolates, I sought to develop an animal model for *C. diphtheriae* infection. No current animal model of corynebacterial infection exists, and an alternative to the mouse model must be used because mice do not have functional diphtheria toxin receptors. Therefore, I chose to use *C. elegans* as a model host for *C. diphtheriae* pathogenesis. The literature has proven that the *C. elegans* infection model can be used to study a variety of bacterial species giving insight into bacterial virulence and host-pathogen interactions (22, 30, 31). In addition to the large array of literature suggesting *C. elegans* can be used to study bacterial pathogenesis, *C. elegans* is also an attractive model host because it is easily maintained and manipulated in the laboratory. For these reasons I sought to study *C. diphtheriae* in the *C. elegans* model system.

To examine whether *C. elegans* can be used as an infection model for *C. diphtheriae*, I first wanted to perform a standard *C. elegans* killing assay with the clinical isolates (Figure 5). The killing assay entails exposing *C. elegans* to two *C. diphtheriae* clinical isolates that lack toxin and all three pilin subunit genes. The percent survival of these nematodes was then compared to the percent survival of nematodes exposed to the type strain corynebacteria, which has toxin and all three major pilin subunit genes. After six days of exposure to the type strain of *C. diphtheriae* all of the worms were dead but when exposed to the clinical isolates, 30-40% of the worms were still alive (Figure 5A). In contrast, when clinical

**Figure 5: *C. diphtheriae* clinical isolates lacking toxin and major pilin subunits are attenuated in the *C. elegans* killing assay.** N2 L4 stage nematodes were exposed to corynebacteria of the type strain (NCTC13129), clinical isolates lacking diphtheria toxin and all three major pilin subunits (CD25/C65 and CD26/C64) (A) or a clinical isolate that has diphtheria toxin and all three major pilin subunits (CD34/749) (B). The number of dead nematodes was recorded daily for 9 days. These data are expressed as percent survival and statistical analysis was performed using GraphPad Prism 5.0 software. The difference between the rates of killing comparing the type strain and clinical isolates lacking toxin and pilin subunits was significant ( $P < 0.05$ ), but rates were not significantly different when comparing the type strain and the isolate that has toxin and major pilin subunits ( $P > 0.05$ ). This experiment was repeated two times with similar results.



isolates that contain *tox* and genes encoding all three major pilin subunits, the rate of killing was comparable to that of the type strain (Figure 5B). These results suggest that corynebacteria lacking pili are attenuated in *C. elegans* killing.

To investigate whether the presence or absence of pilus genes would affect the rate of *C. elegans* killing, the killing assay was also performed with two clinical isolates from each of the seven subgroups and the LT<sub>50</sub> (time it takes for fifty percent of the nematodes to die) of each isolate was determined (Table 3).

Consistent with these data presented in Figure 5, the LT<sub>50</sub> of toxigenic strains (contain *tox*) that have all three major pilin subunit genes (group 7) was comparable to that of the type strain (approximately 3 days). Compared with the type strain and clinical isolates in group 7, all nontoxigenic strains (no *tox*) displayed slower kinetics of killing. All clinical isolates that lack one or more major pilin subunit genes were attenuated in *C. elegans* killing in comparison to nematodes exposed to the type strain *C. diphtheriae*. However, from these data alone, there was no clear distinction that clinical isolates expressing one type of pilus are more pathogenic than others. While toxin and pili appear to be two virulence determinants that contribute to the killing of the nematode, it is not clear from these data which pili contributes the most in pathogenesis. Further analysis of with isogenic *C. diphtheriae* mutants is necessary to determine if a specific pilus-type is a main virulence factor in the *C. elegans* model host.

**Table 3: *C. diphtheriae* clinical isolates in groups 1 through 6 are attenuated in nematode killing compared to the type strain and isolates in group 7.**

Representative *C. diphtheriae* clinical isolates from the seven groups are shown here with the genotypic analysis of *tox* and the genotypic and phenotypic each of the major pilin subunits, *spaA*, *spaD*, and *spaH*. The results of *C. elegans* killing assays are also shown as LT<sub>50</sub> (the time it takes for fifty percent of the nematode population fed on a corynebacterial strain to die) in days.

Origin A	Strain	Biotype <sup>B</sup>	tox analysis				spaA analysis				spaD analysis				spaH analysis				LT <sub>50</sub> , Days																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
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<sup>b</sup> M, *mitis*; B, *belfanti*; G, *gravis*; I, *intermedius*.

<sup>c</sup> Previously published data confirming the toxigenicity of all strains by standard PCR (18).

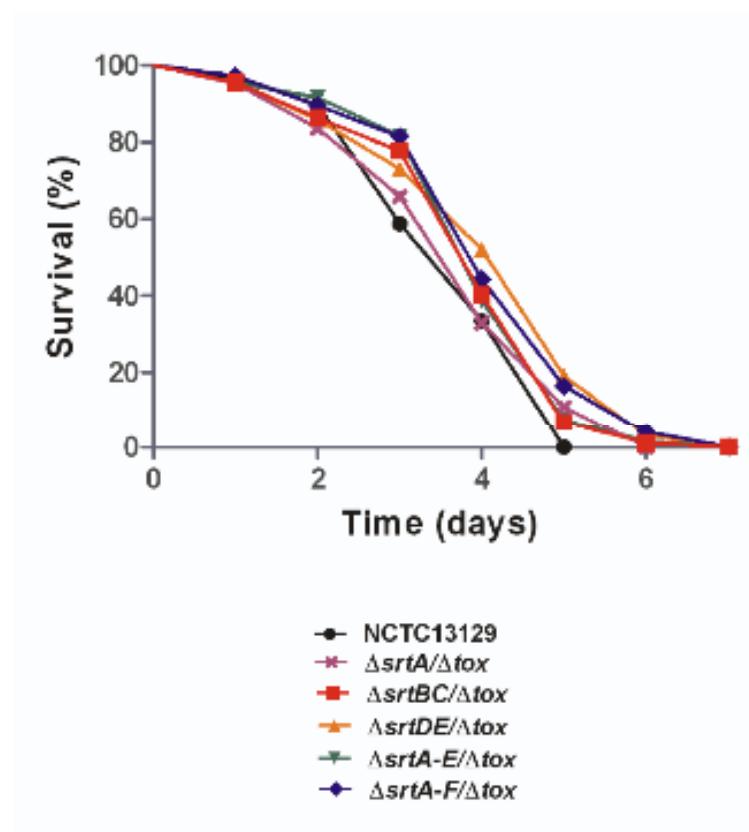
## **Isogenic *Corynebacterium diphtheriae* strains lacking pili are attenuated in nematode killing**

The genetic background of the clinical isolates likely varies from our type strain, which makes direct strain-to-strain comparisons very difficult. For this reason, I sought to test isogenic strains of *C. diphtheriae* in our *C. elegans* model. In order to determine which pilus components are important in pathogenesis, mutant strains lacking various pilus genes as well as diphtheria toxin were made and tested in the nematode assays. The background isogenic mutant strains used in this study were previously generated by the Ton-That laboratory. To exclude the role of toxin, I deleted the *tox* gene in these strains (Table 4A). As discussed in the first chapter, pilus-specific sortase enzymes catalyze the crosslinking of individual pilin monomers and the housekeeping sortase is responsible for anchoring pili to the cell wall. Deletion of a pilus-specific sortase abrogates pilus polymerization. Tested strains include a deletion mutant lacking the SpaA-type pilus-specific sortase ( $\Delta srtA$ ), a deletion mutant lacking the SpaD-type pilus-specific sortases ( $\Delta srtBC$ ), a deletion mutant lacking the SpaH-type pilus-specific sortases ( $\Delta srtDE$ ), a deletion mutant lacking all pilus-specific sortases ( $\Delta srtA-E$ ), and a deletion mutant lacking all six sortases, i.e. pilus-specific sortases and the housekeeping sortase ( $\Delta srtA-F$ ). These *C. diphtheriae* sortase mutants were examined with the *C. elegans* killing assay described above. The results in Figure 6 show that the rates of nematode killing when *C. elegans* were exposed to mutant *C. diphtheriae* was significantly slower as compared to that of *C. elegans* exposed to the parental strain.

**Table 4: Isogenic *C. diphtheriae* strains used in this study.**

Isogenic Mutants		
	Strain	Phenotype
A.	MMB5	$\Delta srtA/\Delta tox$
	MMB6	$\Delta srtBC/\Delta tox$
	MMB2	$\Delta srtDE/\Delta tox$
	MMB3	$\Delta srtA-E/\Delta tox$
	MMB4	$\Delta srtA-F/\Delta tox$
B.	XM1	$\Delta spaABC/\Delta tox$
	XM11	$\Delta spaDEF/\Delta tox$
	XM6	$\Delta spaABCDEF/\Delta tox$
	XM12	$\Delta spaABCDEFGHIG/\Delta tox$

**Figure 6: *C. diphtheriae* isogenic strains lacking various sortase enzymes are attenuated in the *C. elegans* killing assay.** N2 L4 stage nematodes were exposed to corynebacteria of the parent strain or mutant strains lacking various sortases. The number of dead nematodes was recorded daily for 7 days. These data are expressed as percent survival and statistical analysis was performed using GraphPad Prism 5.0 software. The difference between the rate of killing comparing the parental strain to each mutant *C. diphtheriae* strain was significant ( $P < 0.05$ ) in all cases except when comparing the  $\Delta srtA/\Delta tox$  mutant to the parental strain ( $P > 0.05$ ). Rates of nematode killing were not significantly different when comparing each of the various mutant strains to one another ( $P > 0.05$ ). This experiment was repeated two times with similar results.

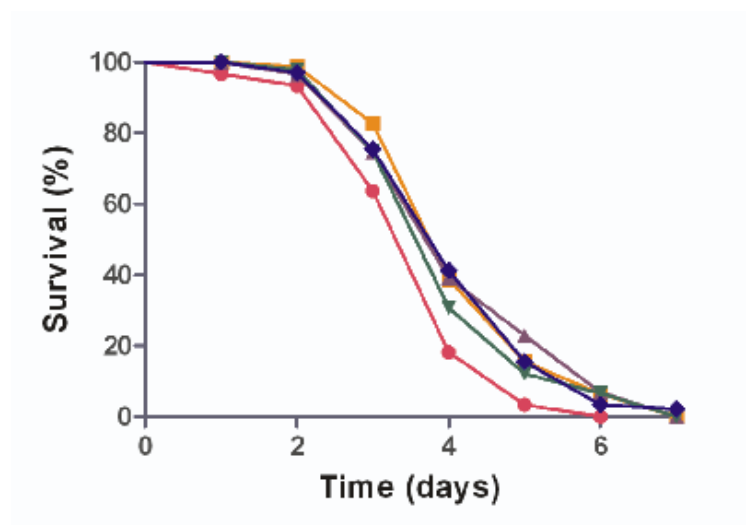


However, when comparing each mutant strain to one another the rates of killing were indistinguishable. It is noteworthy that although no pili are assembled on the surface of sortase deletion mutants, pilin precursors are still made, likely secreted to the extracellular milieu. Therefore, a direct correlation between a specific type of *C. diphtheriae* pilus and pathogenesis in the *C. elegans* model system cannot be made with these mutants. This also suggests that if *C. diphtheriae* is lacking one pilus component, other components may be able to compensate, at least to some degree, in pathogenesis.

As a result of pilin precursors still made in the sortase deletion mutants, mutants that lack individual pilus operons were generated (Table 4B). These include a deletion mutant lacking SpaA-type pili ( $\Delta spaABC/\Delta tox$ ), a deletion mutant lacking the SpaD-type pili ( $\Delta spaDEF/\Delta tox$ ), a deletion mutant lacking SpaA- and SpaD-type pili ( $\Delta spaABCDEF/\Delta tox$ ), and a deletion mutant lacking all pilins ( $\Delta spaABCDEFGHIG/\Delta tox$ ).

These mutants were examined in the *C. elegans* killing assay (Figure 7). Like the sortase mutants, each pilus deletion mutant exhibited a significant reduction in the rate of nematode killing as compared to that of the parental strain. However, there is no significant difference when comparing each mutant to one another. Together these data indicates that corynebacteria lacking pili are attenuated in *C. elegans* killing. These data also indicate that other virulence factors are involved in nematode killing because the *C. elegans* exposed to the mutant corynebacteria are not completely attenuated.

**Figure 7: *C. diphtheriae* isogenic strains lacking various pilins are attenuated in the *C. elegans* killing assay.** N2 L4 stage nematodes were exposed to corynebacteria of the parent strain or mutant strains lacking various pili. The number of dead nematodes was recorded daily for 7 days. These data are expressed as percent survival and statistical analysis was performed using GraphPad Prism 5.0 software. The difference between the rate of killing comparing the parental strain and each mutant *C. diphtheriae* strain was significant ( $P < 0.05$ ), but rates were not significantly different when comparing each various mutant strain to one another ( $P > 0.05$ ). This experiment was repeated two times with similar results.



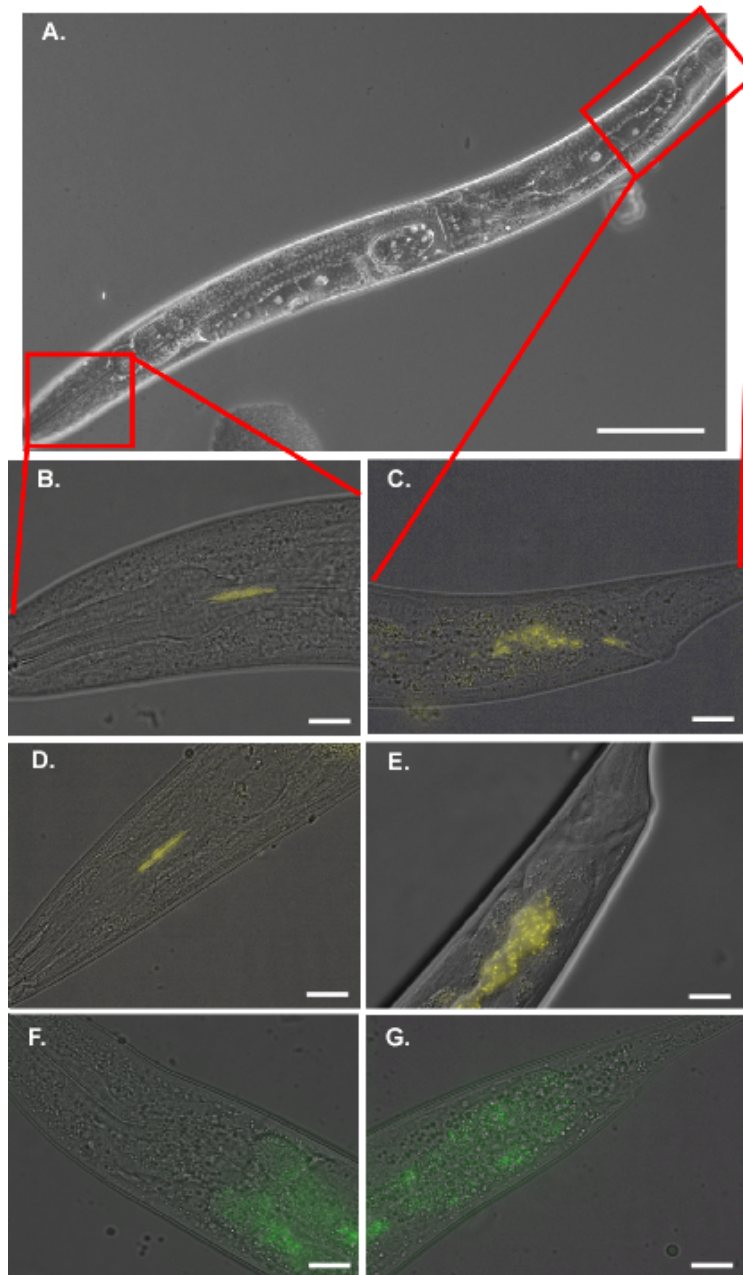
- NCTC13129
- $\Delta spaABC/\Delta tox$
- ▲—  $\Delta spaDEF/\Delta tox$
- ▼—  $\Delta spaABCDEFGH/\Delta tox$
- ◆—  $\Delta spaABCDEFGHIG/\Delta tox$

## ***Corynebacterium diphtheriae* infection localizes to *Caenorhabditis elegans* pharynx and hind gut**

To examine the corynebacterial infection directly in *C. elegans*, which is a transparent organism, I utilized fluorescence microscopy. In order to view *C. diphtheriae*, pK-PIM, an integration vector (23) containing yellow fluorescent protein (YFP) under the control of a constitutive promoter, was used. This vector was delivered to *C. diphtheriae* via conjugation from *E. coli* and integrated into the corynebacterial chromosome at *attP* sites (23). The nematodes were then exposed to the fluorescent *C. diphtheriae* for different periods of time and visualized under the microscope.

In Figure 8A, the full size nematode and the areas with the most *C. diphtheriae* localization are highlighted with red boxes. These areas include the pharynx region (Figure 8B, 8D, and 8F) and the hindgut (Figure 8C, 8E, and 8G). Figure 8, B and C depict a worm that was exposed to *C. diphtheriae* for 18 hours, while D and E depict a worm that was exposed to *C. diphtheriae* for 24 hours. From 18 to 24 hours, there was an accumulation of *C. diphtheriae* in the hindgut (C vs. E), while the levels of *C. diphtheriae* in the pharynx remained the same (B and D). As a control I also observed *C. elegans* exposed to non-pathogenic *E. coli* OP50, the nematode lab diet (Figure 8F and 8G). *C. elegans* contain auto fluorescent lipofuscin granules that appear green in these images. In addition to *C. diphtheriae* localization; there is a stark difference in tail swelling when nematodes are fed on *C.*

**Figure 8: *C. diphtheriae* NCTC13129 causes gut distention and tail sllling in *C. elegans*.** N2 L4 stage worms were exposed to *C. diphtheriae* NCTC13129 strain for 18 (B, C) and 24 hours (D, E) and to OP50 non-pathogenic *E. coli* for 24 hours (F, G). 18 and 24 hours post-infection worms were obtained, anesthetized and washed to remove residual bacteria. They were then visualized under the microscope. 8A depicts the full size nematode and the areas of *C. diphtheriae* localization (highlighted with red boxes) are the pharynx region (Figure 8B, 8D, and 8F) and the hindgut (Figure 8C, 8E, and 8G). From 18 to 24 hours, there was an accumulation of *C. diphtheriae* in the hindgut (C vs. E), while the levels of *C. diphtheriae* in the pharynx remained the same (B and D). As a control I also observed *C. elegans* exposed to normal lab diet (F and G). *C. elegans* contain auto fluorescent lipofuscin granules that appear green in these images (F and G). Bars indicate a distance of 100  $\mu\text{m}$ .



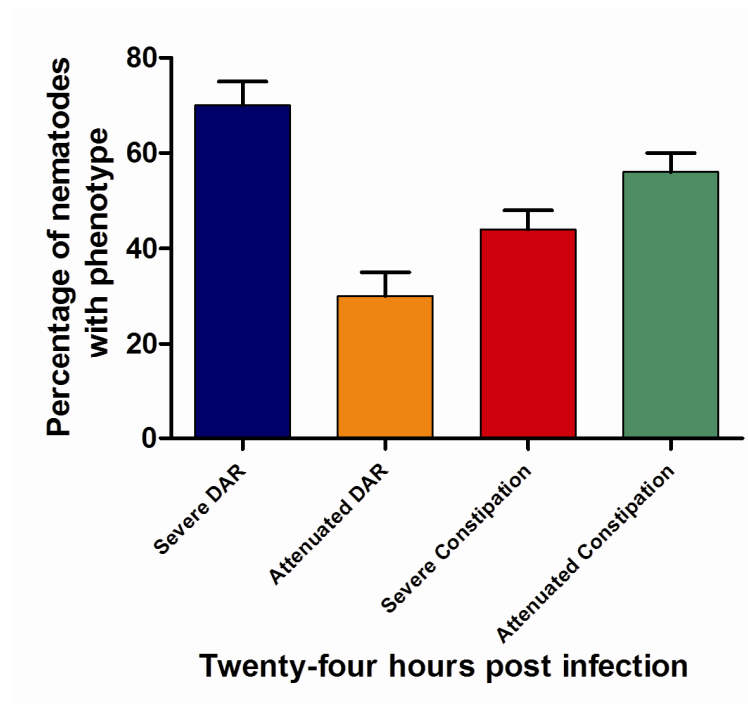
*diphtheriae* compared to feeding on *E. coli* OP50, and nematodes fed on *C. diphtheriae* also exhibit a distended gut. The significant tail swelling that occurs in nematodes infected with *C. diphtheriae*, is seen in Figure 8C and 8E where the tail does not come to a point as it does when the nematodes are exposed to *E. coli* OP50 (Figure 8G). The tail swelling phenotype is called deformed anal region or Dar and has only been observed when *C. elegans* are exposed to one other species, *M. nematophilum* (7).

### ***Corynebacterium diphtheriae* infection mediates Dar formation and constipation in *Caenorhabditis elegans***

The Dar phenotype was first observed by the Hodgkin group and they found that *M. nematophilum* adheres to the rectal cuticle of the nematodes and causes swelling of the underlying tissue, but the infection is not lethal (7). The tail swelling occurs as a result of an immune response to combat nematode constipation caused by the bacterial infection (21). I investigated whether the same phenotypes, Dar and constipation, are observed when *C. elegans* are exposed to *C. diphtheriae*. At 24 hours post-infection, approximately 70% of the nematodes examined express the Dar phenotype and 45% of the nematodes were severely constipated (Figure 9); severe constipation was also accompanied by a distention in the later part of the intestine, close to the anus of the nematode.

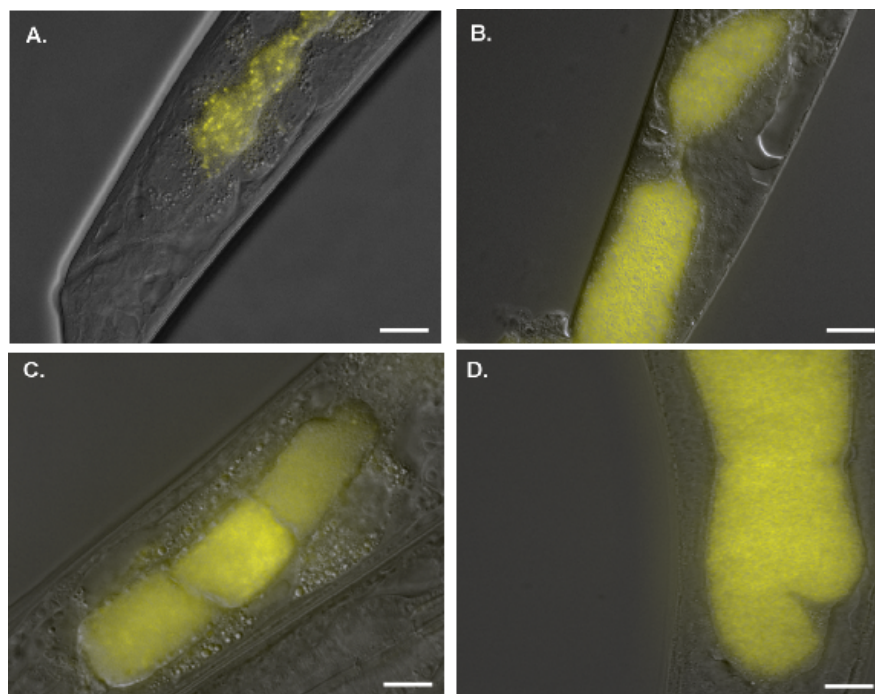
A time course assay was also performed to visually analyze the *C. diphtheriae* infection in the nematodes at 24, 48, 72, and 96 hours post-infection (Figure 10). The LT<sub>50</sub> of nematodes exposed to parental *C. diphtheriae* is

**Figure 9: 24 hours post-infection with *C. diphtheriae* NCTC13129, 70% of *C. elegans* express Dar phenotype and 45% are severely constipated.** N2 L4 stage worms were exposed to *C. diphtheriae* NCTC13129 for 24 hours, anesthetized, washed to remove residual bacteria, and visualized by microscopy. Severe Dar compared to attenuated Dar (refer to Figure 8E compared to 8G) is when the tail does not come to a point as it does when the nematodes are exposed to *E. coli* OP50. Severe constipation compared to attenuated constipation (refer to Figure 8E compared to 8C) is when the hind gut of the worm is distended and full of corynebacteria.



**Figure 10: Time course assay following *C. diphtheriae* infection in *C. elegans*.**

N2 L4 stage worms were exposed to *C. diphtheriae* NCTC13129 strain for 24 (A), 48 (B), 72 (C), or 96 hours (D). The nematodes were anesthetized, washed to remove residual bacteria, and visualized by microscopy. The severity of *C. elegans* constipation and gut distension increases over time and appears to move up the nematodes body from the anus to the middle intestine. Bars indicate a distance of 100  $\mu\text{m}$ .



approximately three days; therefore with this time course I would be able to see the infection as it progresses from initial colonization to fatal illness in the nematode. As shown in Figure 10A, 24 hours post-infection, severe constipation and hindgut distention were observed. 48 hours post-infection (Figure 10B), constipation appeared to increase and corynebacterial colonization expanded towards the middle of the nematode. Interestingly, segmented pod-like clusters were observed within the nematode gut. 72 hours post-infection (Figure 10C), the constipation worsened and the pod-like clusters came together. It is clear from these images that the nematode was unable to excrete the *C. diphtheriae* and the bacteria were building up in the *C. elegans* intestine. 96 hours post-infection, *C. elegans* were almost completely full of *C. diphtheriae*. The intestine was distended to the full width of the nematode, filled with *C. diphtheriae* (Figure 10D). Whether or not corynebacterial pili are involved in the Dar and constipation phenotypes is being investigated. When studying these *C. diphtheriae* mutants the Dar phenotype can be used as a visual marker to examine strains unable to elicit this immune response in the *C. elegans* model host.

## **Chapter 4: Discussion**

Infection from *C. diphtheriae* can cause airway obstruction and eventually lead to death. Prior to widespread vaccination, diphtheria epidemics were known to kill up to 40% of infected patients. Today, however, diphtheria has largely been controlled due to vaccination (19). Nevertheless, outbreaks of diphtheria still occur worldwide. Furthermore, incidents of infections caused by non-toxigenic strains of *C. diphtheriae* have been increasingly reported (15, 26). Thus it is prudent that the mechanisms of *C. diphtheriae* infection be investigated thoroughly and that has been the focus of this study.

This work has focused on pili as a potential virulence factor of *C. diphtheriae*. Bacterial pili are proteinaceous fibers attached to the cell surface which may aid in host colonization. In this study, I have characterized a large collection of *C. diphtheriae* clinical isolates obtained from the CDC to analyze them for the distribution of pilus gene clusters. Based on the presence or absence of toxin and pilus genes, I placed 46 clinical isolates into 7 sub-groups. Groups 2 and 3 which have been characterized as non-toxigenic, as has group 1 (Table 1), contain no toxin but at least one major pilin subunit gene. Additionally, most isolates expressed at least one of the major pilin subunits (61%), with SpaA being expressed in all of these isolates. This suggests that Spa proteins play a role during adherence and infection. It is interesting to note that there is a wide range of clinical isolates containing different pilin subunits in the presence or absence of toxin. This suggests that there is great genetic diversity within *C. diphtheriae* clinical isolates.

Future work with these clinical isolates will be to characterize them further for the presence and expression of minor pilin subunits, as well as, for their ability to

bind to human pharyngeal cells. Previous work has shown that minor pilin subunits are the major adherence factors required for *C. diphtheriae* to adhere to host pharyngeal cells (12). It would be interesting to see if the corynebacterial clinical isolates lacking major pilin subunits still expressed minor pilin subunits that might bind host cells. It is possible that possessing minor pilin subunits on the surface of *C. diphtheriae* is enough to moderate binding to host cells.

The *C. elegans* killing assay was used to determine if a correlation between pilus expression in *C. diphtheriae* clinical isolates and increased *C. elegans* lethality exists. I have shown that isolates lacking toxin and major pilin subunits are attenuated in nematode killing, compared to isolates that have toxin and all three major pilin subunits or the type strain. Because the genetic background of these isolates varies, it is not possible to determine whether the presence of one pilus type would enhance the rate of killing by these isolates.

Considering unknown genetic differences between the clinical isolates, I next examined isogenic strains in the *C. elegans* model. I tested two groups of isogenic *C. diphtheriae* mutants, one lacking various sortase enzymes and the other lacking various pilus types. Both groups of *C. diphtheriae* mutants were significantly attenuated in nematode killing compared to that of the parental strain. However, the differences are not so drastic that the role of individual pili is clear using the *C. elegans* killing assay. There are a number of possible explanations for this. It is possible that pili are not expressed in the worm, or that the nematodes may not have receptors for corynebacterial pili. The human receptor for corynebacterial pili is unknown. Further experiments are needed to determine if toxin and pili are

produced in the worm during infection. It may be possible to homogenize nematodes exposed to *C. diphtheriae* and precipitate proteins in that homogenized solution in order to perform western blot analysis looking for pilus protein expression. The same type of experiment can also be performed looking for diphtheria toxin expression. These types of experiments would solidify that toxin and pili are being produced during *C. elegans* infection with *C. diphtheriae*.

Although toxin has been shown to be the causative agent of diphtheria and pili are the major adhesions for host pharyngeal cells, neither factor was shown to be critical for lethality in the *C. elegans* model host. This may be a limitation of the *C. elegans* model for corynebacterial infection. Therefore, it may also be necessary to elucidate a vertebrate animal model, such as the guinea pig, that has a DT receptor, to fully study corynebacterial pathogenesis.

Nevertheless, the *C. elegans* system allows us to visualize bacterial colonization inside a host in order to elucidate the host-pathogen relationship. I have shown *C. diphtheriae* colonizes at the nematode pharynx region and the hindgut. In addition to this localization, the bacteria accumulate over time in the hindgut of the worm when the nematodes are fed on *C. diphtheriae*.

Furthermore, worms exposed to *C. diphtheriae* show significant tail swelling, a phenotype known as deformed anal region (Dar). The tail swelling occurs as a result of the nematode immune response to bacterial infection. The immune response, and subsequent Dar formation, is combating constipation caused by the pathogen. Nematode constipation and Dar phenotypes are accompanied by a distended gut (7). A time-course of infection from 24 to 96 hours post-infection

demonstrates that the nematode is literally filling up with corynebacteria. *C. elegans* exposed to *C. diphtheriae* for 48 hours appear to contain pod-like clusters of bacteria forming in the nematode gut. The pods consist of a large cluster of bacteria, followed by a thin segment of the worm intestine, followed by a large cluster of bacteria. At 72 hours post-infection, these pods have grown so large that the thin segments are no longer visible between clusters of *C. diphtheriae*. It is possible that these pods are similar to a series of biofilms being formed within the *C. elegans* intestine. Biofilms can be defined as communities of microorganisms that are attached to a surface forming a microcolony (24). In biofilm formation, bacteria genetically regulate transitioning from a planktonic cell to attachment to a surface, colonization of that surface and formation of a microcolony (17). In order to further investigate the pod phenotype I have observed in this study, it will be necessary to screen *C. diphtheriae* mutants defective in pod formation to see if there is in fact a biofilm being formed within the nematode intestine.

It is conceivable that when the nematode encounters *C. diphtheriae* infection, the animal develops the Dar to prevent bacterial colonization. The pathogen counters this strategy by generating blockage of the nematode intestinal tract to prevent excretion. As this occurs, corynebacteria are able to grow and expand their colonies throughout the animal intestine. Whether pili are required for these processes remains to be investigated. Future experiments are needed to test the isogenic *C. diphtheriae* pili mutants for nematode blockage and the Dar phenotype. An advantage of using *C. elegans* as a model host is that large numbers of mutants can be screened relatively easily, thus allowing for the study of not only isogenic

mutants but also a *C. diphtheriae* transposon library. This will elucidate other virulence factors *C. diphtheriae* may possess.

In conclusion, I demonstrate for the first time that *C. elegans* can be used as an animal model for the study of *C. diphtheriae* pathogenesis. *C. diphtheriae* clinical isolates were tested for the presence and expression of diphtheria toxin as well as all three *C. diphtheriae* major pilin subunits. I found that in these clinical isolates all three major pilin subunits as well as toxin are required for rapid nematode killing. I observed *C. elegans* fed on *C. diphtheriae* show significant tail swelling (Dar). This immune response to infection by *C. elegans* can be used in the future to study other aspects of *C. diphtheriae* pathogenesis and identify additional virulence factors.

## References

1. **Cerdeno-Tarraga, A. M., A. Efstratiou, L. G. Dover, M. T. Holden, M. Pallen, S. D. Bentley, G. S. Besra, C. Churcher, K. D. James, A. De Zoysa, T. Chillingworth, A. Cronin, L. Dowd, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Moule, M. A. Quail, E. Rabinowitsch, K. M. Rutherford, N. R. Thomson, L. Unwin, S. Whitehead, B. G. Barrell, and J. Parkhill.** 2003. The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. *Nucleic Acids Res* **31**:6516-23.
2. **de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using lacIq/P<sub>trp</sub>-lac plasmids and transposons that confer conditional phenotypes. *Gene* **123**:17-24.
3. **Garsin, D. A., C. D. Sifri, E. Mylonakis, X. Qin, K. V. Singh, B. E. Murray, S. B. Calderwood, and F. M. Ausubel.** 2001. A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci U S A* **98**:10892-7.
4. **Gaspar, A. H., and H. Ton-That.** 2006. Assembly of distinct pilus structures on the surface of *Corynebacterium diphtheriae*. *J Bacteriol* **188**:1526-33.
5. **Hadfield, T. L., P. McEvoy, Y. Polotsky, V. A. Tzinserling, and A. A. Yakovlev.** 2000. The pathology of diphtheria. *J Infect Dis* **181 Suppl 1**:S116-20.
6. **Hodgkin, J.** 2004. Dissecting worm immunity. *Nat Immunol* **5**:471-2.
7. **Hodgkin, J., P. E. Kuwabara, and B. Corneliussen.** 2000. A novel bacterial pathogen, *Microbacterium nematophilum*, induces morphological change in the nematode *C. elegans*. *Curr Biol* **10**:1615-8.

8. **Hodgkin, J., and F. A. Partridge.** 2008. *Caenorhabditis elegans* meets microsporidia: the nematode killers from Paris. *PLoS Biol* **6**:2634-7.
9. **Hope, I. A.** 1999. *C. elegans: A Practical Approach*. Oxford University Press, Inc., New York City.
10. **Love, J. F., and J. R. Murphy.** 2006. *Corynebacterium diphtheriae*: Iron-mediated activation of DtxR and regulation of diphtheria toxin expression, p. 726-737. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), *Gram-positive pathogens*, Second Edition ed. ASM Press, Washing D.C.
11. **Mandlik, A., A. H. Gaspar, A. Swaminathan, A. Mishra, A. Das, and H. Ton-That.** 2009. Gram-positive Bacterial Pili and the Host–Pathogen Interface, p. 75-90. *In* K. F. Jarrell (ed.), *Pili and Flagella: Current Research and Future Trends*. Caister Academic Press, Norfolk, UK.
12. **Mandlik, A., A. Swierczynski, A. Das, and H. Ton-That.** 2007. *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. *Mol Microbiol* **64**:111-24.
13. **Mandlik, A., A. Swierczynski, A. Das, and H. Ton-That.** 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. *Trends Microbiol* **16**:33-40.
14. **Marraffini, L. A., A. C. Dedent, and O. Schneewind.** 2006. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol Mol Biol Rev* **70**:192-221.

15. **Mattos-Guaraldi, A. L., L. O. Moreira, P. V. Damasco, and R. Hirata Junior.** 2003. Diphtheria remains a threat to health in the developing world--an overview. *Mem Inst Oswaldo Cruz* **98**:987-93.
16. **Mazmanian, S. K., G. Liu, H. Ton-That, and O. Schneewind.** 1999. Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. *Science* **285**:760-3.
17. **Monds, R. D., and G. A. O'Toole.** 2009. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol* **17**:73-87.
18. **Mothershed, E. A., P. K. Cassidy, K. Pierson, L. W. Mayer, and T. Popovic.** 2002. Development of a real-time fluorescence PCR assay for rapid detection of the diphtheria toxin gene. *J Clin Microbiol* **40**:4713-9.
19. **Murphy, J. R.** 1996. *Corynebacterium diphtheriae*. In S. Baron (ed.), *Medical Microbiology*, 4 ed. The University of Texas Medical Branch, Galveston.
20. **Nakao, H., and T. Popovic.** 1997. Development of a direct PCR assay for detection of the diphtheria toxin gene. *J Clin Microbiol* **35**:1651-5.
21. **Nicholas, H. R., and J. Hodgkin.** 2004. The ERK MAP kinase cascade mediates tail swelling and a protective response to rectal infection in *C. elegans*. *Curr Biol* **14**:1256-61.
22. **Nicholas, H. R., and J. Hodgkin.** 2004. Responses to infection and possible recognition strategies in the innate immune system of *Caenorhabditis elegans*. *Mol Immunol* **41**:479-93.

23. **Oram, M., J. E. Woolston, A. D. Jacobson, R. K. Holmes, and D. M. Oram.** 2007. Bacteriophage-based vectors for site-specific insertion of DNA in the chromosome of *Corynebacteria*. *Gene* **391**:53-62.
24. **O'Toole, G., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* **54**:49-79.
25. **Riddle, D. L., T. Blumenthal, B. J. Meyer, and J. R. Priess.** 1997. *C. elegans* II, 2 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
26. **Romney, M. G., D. L. Roscoe, K. Bernard, S. Lai, A. Efstratiou, and A. M. Clarke.** 2006. Emergence of an invasive clone of nontoxigenic *Corynebacterium diphtheriae* in the urban poor population of Vancouver, Canada. *J Clin Microbiol* **44**:1625-9.
27. **Rozen, S., and H. J. Skaletsky** 2000, posting date. Primer3 on the WWW for general users and for biologist programmers. Humana Press. [Online.]
28. **Saragea, A., P. Maximescu, and E. Meitert.** 1979. *Corynebacterium diphtheriae*: Microbiological Methods Used in Clinical and Epidemiological Investigations, p. 62-138. *In* T. Bergan and J. R. Norris (ed.), *Methods in Microbiology*, vol. 13. Academic Press, Inc., New York City.
29. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**:69-73.

30. **Schulenburg, H., and S. Muller.** 2004. Natural variation in the response of *Caenorhabditis elegans* towards *Bacillus thuringiensis*. *Parasitology* **128**:433-43.
31. **Sifri, C. D., J. Begun, and F. M. Ausubel.** 2005. The worm has turned-- microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol* **13**:119-27.
32. **Telford, J. L., M. A. Barocchi, I. Margarit, R. Rappuoli, and G. Grandi.** 2006. Pili in Gram-positive pathogens. *Nat Rev Microbiol* **4**:509-19.
33. **Ton-That, H., and O. Schneewind.** 2003. Assembly of pili on the surface of *Corynebacterium diphtheriae*. *Mol Microbiol* **50**:1429-38.
34. **Wood, W. B.** 1988. Introduction to *C. elegans* Biology, The nematode *C. elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
35. **Yanagawa, R., and E. Honda.** 1976. Presence of pili in species of human and animal parasites and pathogens of the genus *Corynebacterium*. *Infect Immun* **13**:1293-5.

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