Identification and Characterization of Inerolysin, the Cholesterol Dependent Cytolysin produced by *Lactobacillus iners*

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Abstract

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Lactobacillus iners, is a gram positive organism recently identified through the use of culture independent techniques. Identified as a major constituent of the vaginal microbiota, epidemiological studies have suggested that this organism may not provide the protective effects ascribed to other vaginal lactobacilli. Our work here has identified and characterized a pore forming toxin, Inerolysin, produced by this organism. This pore forming toxin was present in all strains of *L. iners* tested and possessed characteristics which firmly categorize it as a member of the Cholesterol Dependent Cytolsin superfamily.

Additionally, we identified pH as a regulatory factor for the activity of Inerolysin as well as other CDCs. Inerolysin was shown to had optimal activity at acidic pH, while other toxins such as pneumolysin and arcanolysin had optimal activity at basic pH. We demonstrate that pH induced changes in activity were reversible, suggesting that a reversible conformational change takes place in the protein. Furthermore, our results show that it is the last step in pore formation, the transition from pre-pore to pore, which is impaired. Our attempts to localize this to particular residues were unsuccessful.

Finally, we sought to understand what the vaginal environment of a Lactobacillus dominated flora would look like. We demonstrate that *L. iners* induced unique signaling in the vaginal epithelium, leading to the production of a unique profile of proinflammatory cytokines as well as antimicrobial peptides. We further demonstrate that some of these responses are mediated by the activity of the pore forming toxin of *L. iners*, Inerolysin.

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Chapter 1: Introduction

1.1 Normal Microbiota of the Vaginal Tract

The microbial inhabitants of the female genital tract and the contribution of these organisms to health and disease have been investigated for well over a century, yet they remain incompletely understood. The first studies which yielded information about the complexity of this anatomical niche were focused on determining whether puerperal sepsis (also known as childbed fever, a bacterial infection acquired during childbirth) resulted from the contamination of normally sterile sites or whether the necessary agents already existed in the vagina. In 1887, Döderlein attempted to address this issue by examining the vaginal secretions and uterine cavity samples of pregnant women[1, 2]. He found that uterine samples were sterile, but vaginal secretions frequently contained pyogenic cocci. This work was important in demonstrating the presence of bacterial species in the vaginal tract, and more importantly introduced us to the first descriptions and images of lactobacilli, which was referred to as the Döderlein bacillus (and would later be identified as *Lactobacillus acidophilus)[3]*.

These early studies were dependent upon the growth of these bacteria in rich media and identification and characterization based on observable characteristics (shape, Gram stain, and arrangement of cells). With advances in the bacteriological culture strategies, scientific understanding of the vaginal microbiota became significantly more nuanced. While yielding important information about the composition of the colonizing microbiota, this approach was inherently biasing the picture obtained, as only a small fraction of the colonizing microbiota

could be cultured in the laboratory, either because they could not grow on traditional media or because they were present in such low abundance.

In the 1980's, several groups demonstrated that the phylogenetic relationships of several bacteria could be determined by the comparison of a stable part of the genetic code. The 16S rRNA gene was chosen because of its length (large enough to be sequenced directly) and because the amount of sequence change is a product of the rate of change and the time (so greater sequence change correlates to the phylogenetic distance of two sequences since they diverged from their last common ancestor)[4]. Contained within the 16SrRNA gene are "hot spots" that show larger number of mutations. As our understanding grew, it became clear that these short regions could also yield significant phylogenetic information and allow for a higher resolution between species (as these regions contained more changes between species). Briefly, primers are generated to the conserved regions of the 16S rRNA gene, producing a polymerase chain reaction (PCR) product that spans one or more of these hypervariable regions. These products can then be analyzed in several ways to assess community structure. These methods include terminal restriction fragment length polymorphism (T-RFLP) analysis, in which PCR products are generated and digested with restriction enzymes, yielding patterns of fragments used for identification. The PCR products may also be analyzed by denaturing gradient gel electrophoresis (DGGE) in which they are separated based on sequence differences by subjecting them to the activity of a gradient of denaturing chemicals. Whereas T-RFLP and DGGE are useful and economical techniques, direct sequencing of 16S PCR products yields the most detailed information and, as sequencing costs have decreased, has become the most commonly used technique. The recent transition from low-throughput clone library sequencing

studies to deep sequencing of PCR amplicons has led to a rapid accumulation of data regarding human-associated microbial communities and has been crucial in furthering our understanding of the composition and dynamics of the microbiota of the genital tract and their role in health and disease[5-8].

As deep sequencing has become more cost effective and commonplace, we have gained further insight into the diversity that is present between individuals. One study demonstrated that rather than having a core set of microbial species present in the vaginal tract common to all women, there were actually five "community state types", characterized by the dominant species present[9]. This work found that community state types I, II, III, and V were dominated by a single vaginal *Lactobacillus* species (either *L. crispatus, L. gasseri, L. iners, and L. jensenii* respectively) while community state type IV was characterized by a high diversity and no single dominant *Lactobacillus* species. This work and others utilizing culture-independent techniques have greatly expanded the census of vaginal microbes. Among the aerobes and facultative organisms, lactobacilli, other Gram-positive rods, staphylococci and streptococci (of both pathogenic and non-pathogenic varieties), and Gram-negative enteric organisms can all be found at high prevalence in vaginal samples. Likewise, a variety of cultivable anaerobes including *Prevotella* spp., *Fusobacteria* spp., and others are present in substantial numbers at the vaginal mucosal surface[10]. The identification of the high level of diversity in the vaginal tract is a first step, and further work is required to understand how these species contribute to vaginal health and disease[11].

1.1.1 Role of "Protective" Lactobacilli in the Vaginal Tract

Döderlein's work not only established the dominance of vaginal lactobacilli in the vaginal tract, but established an important concept with regards to their role in the vaginal tract. In his study of puperal sepsis he suggested that in normal secretions, vaginal lactobacilli were crucial to keeping the vagina free of pathogenic bacteria[2]. This crucial concept continued to inform studies into the 20th century, and it was later demonstrated that the growth of *Lactobacillus acidophilus* antagonized the growth of *Neisseria gonorrhoeae*[12]. It has been suggested that the protective effects of vaginal lactobacilli can be attributed to several mechanisms, including the production of hydrogen peroxide[13] and lactic acid (that maintains the vaginal tract at an acidic pH), the production of bacteriocins (small peptides with microbicidal activity) and competition for nutrients or receptors at the epithelial cell surface, a phenomenon known as bacterial interference. There has been significant work in understanding the protective effects of these organisms and their specific identity in the hopes of developing various probiotics for the treatment and prevention of urogenital infections.

1.2 Bacterial Vaginosis

Bacterial vaginosis (BV) is a condition characterized by replacement of the normally protective *Lactobacillus* spp. and a massive overgrowth of anaerobic and facultative organisms including *Gardnerella vaginalis, Atopobium vaginae, Bacteroides spp., Mobiluncus spp*., and genital mycoplasmas[14, 15]. With the loss of the *Lactobacillus* species, we also see an elevation of the vaginal pH due to their lactic acid producing capability[16, 17]. This alteration in vaginal microbiology may lead to symptomatic vaginitis. However, the vast majority of affected women

remain asymptomatic[18]. Regardless of clinical presentation, BV is associated with significant adverse consequences including miscarriage, preterm birth, chorioamnionitis[19], postpartum endometritis and an increased risk of HIV/STD acquisition[12, 20, 21]. This complex disorder is exceedingly common, with prevalence rates ranging from 10% to 40%(reviewed in [22]). Suboptimal methods of diagnosis reflect the inherent difficulties in precisely defining this condition and make the true prevalence difficult to ascertain.

1.2.1 Etiology of Disease

Despite an understanding of the changes that occur during an episode of BV, the etiology of this disease remains unclear and the cause of disease has yet to be identified. Studies of this disease state have yielded several potential candidates as contributing to disease. In 1955, Gardner and Dukes isolated *Haemophilus vaginalis*, now known as *G. vaginalis*, from women with 'nonspecific vaginitis' and postulated that this was the primary etiological agent[23]. Their attempts to induce infection in healthy volunteers by inoculating pure cultures of *G. vaginalis* were largely unsuccessful (only one of 13 volunteers infected)[24]. They were, however, able to induce vaginitis in 11 of 15 volunteers by inoculating vaginal secretions from affected women, suggesting that *G. vaginalis* alone was not sufficient to induce disease and that additional factors/organisms were necessary. Since that time, numerous BV-associated bacteria have been identified using both standard culture and cultivation- independent techniques and BV is generally regarded as a polymicrobial disease. Nevertheless, *G. vaginalis* remains one of the most frequently isolated organisms in women with BV[25] and its cytotoxicity and ability to

produce an adherent biofilm suggests a greater virulence potential relative to other BV-associated organisms[26-28].

Culture based studies have implicated a number of different organisms in the etiology of this disease. Several studies have demonstrated an association between *Mobiluncus curtisii[21]* and BV, with prevalence rates approaching 77% in BV patients as compared to healthy controls where *Mobiluncus* species were identified in about 6% of samples. Several studies have also identified *Mycoplasma hominis* as a highly associated BV organism, being present in 24-75% of women with BV and only 13-22% of women without BV[29-32]

The use of cultivation-independent techniques has further enhanced our understanding of the microbiology of BV. Fredricks et al.[33] used 16S rDNA PCR to characterize and compare the bacterial communities found in women with and without BV. They demonstrated that those subjects with BV exhibited considerably greater bacterial diversity, with 35 bacterial phylotypes detected, 16 of which were newly recognized. Collectively, these organisms, belonging to the *Clostridiales* group, were referred to as BVAB1, BVAB2, and BVAB3[34]. While there is no evidence that these organisms are the causative agents of BV (possibly because of their fastidious nature making them difficult to study in a laboratory setting), they are highly specific markers of BV[35]. By contrast, women without BV were noted to have relatively homogeneous vaginal microbiota, predominantly comprised of lactobacilli. Despite the identification of several BV-associated organisms, a clear link between these species and the onset of a BV episode has not been explicitly demonstrated. Furthermore, these species are also notably present in healthy patients, calling into question whether they are each

individually the cause of this disease. To date, no single organism has been implicated in the development of this disease and the sequence of events in the progression of BV remains unclear. Figure 1.1 shows two competing models for the etiology of bacterial vaginosis[36].

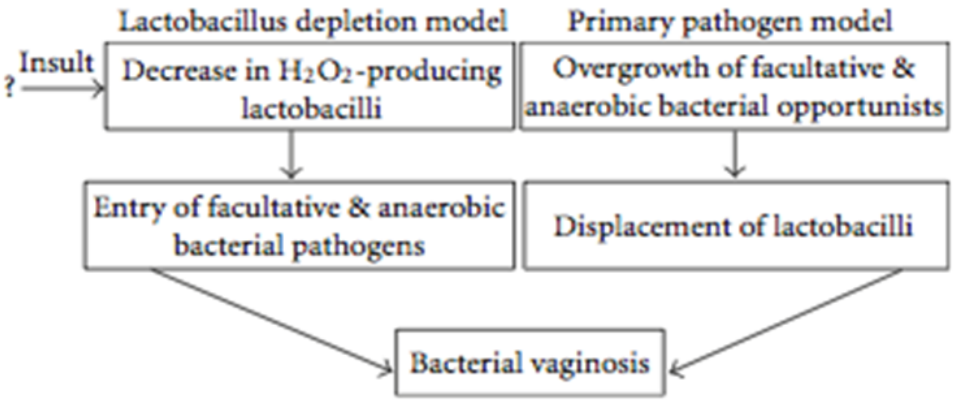


Fig 1.1 Two competing models for the etiology of bacterial vaginosis. In the *Lactobacillus* depletion model, a loss of hydrogen peroxide producing lactobacilli allows an overgrowth of facultative anaerobic bacterial pathogens, leading to a BV state. In the primary pathogen model, an overgrowth of potential pathogens is the initiating factor, resulting in a displacement and depletion of the protective *Lactobacillus* species, leading to bacterial vaginosis. It is still not clear whether an insult is the initiating factor for all of these events, or if the flora is inherently susceptible to these changes.. Adapted from Srinivasan et al 2008 [36]

1.2.2 Diagnosis and Clinical Outcome

Historically, BV was a diagnosis of exclusion. Women who presented with a discharge but lacked detectable yeast cells or *Trichomonas* infection were given the diagnosis of bacterial

vaginosis (then referred to as nonspecific vaginitis). The use of clinical symptoms for diagnosis became more standardized by Amsel[18] who defined BV as having three of the four following clinical signs: (i) vaginal fluid with a pH of >4.5, (ii)A homogenous grey discharge,(iii) fishy odor after the addition of 10% KOH, and (iv) presence of clue cells on wet mount (shed epithelial cells coated with bacterial cells). Since then, these criteria are still in wide use because of the ease with which they can be performed in a clinical setting. However, a number of studies since then have suggested that the use of these subjective criteria can result in misdiagnosis, either seeing BV where it is not, or missing it altogether in other patients. This is particularly concerning for women who have an altered vaginal microbiota but lack observable symptoms, and thus are currently not treated. These women, despite having no frank discharge or other symptoms, could still suffer from some of the negative consequences associated with BV, although the connection between asymptomatic BV and BV associated consequences has not yet been examined.

Alternatively, BV may be diagnosed using the Nugent scoring system for interpretation of Gram-stained vaginal smears[37]. This method assesses the number of lactobacilli relative to BV-associated bacterial morphotypes in order to characterize vaginal microbiota as normal, intermediate or abnormal (BV). A major criticism of this diagnostic strategy is that although women with high numbers of *Lactobacillus spp*. generally do not have BV, it may be incorrect to conclude that women with few or no *Lactobacillus spp*. have BV. In the study by Ravel et al., nearly 25% of asymptomatic reproductive-age women had vaginal bacterial communities in the

'diversity group' (i.e. not dominated by *Lactobacillus spp*.)[9]. Additionally, the data we present here demonstrates that the presence of lactobacilli in a vaginal smear is not synonymous with health, depending on the *Lactobacillus* species present.

In recent years, there has been an increasing appreciation for bacterial vaginosis as an important clinical entity, because of the significant adverse sequelae associated with it. These include an increased risk for pelvic inflammatory disease, increased risk for postpartum endometritis, an increased risk for the acquisition of HIV and other STDs, as well as an increased risk for low birth weight[38] and preterm birth[39-43]

Preterm birth is defined as delivery of an infant before 37 weeks. In the US, it occurs in 11% of all pregnancies, and is associated with high costs (in excess of $4 billion/year) and a high rate of neonatal morbidity and mortality[44]. Various studies have demonstrated the association between BV and the incidence of preterm birth although the exact assessment of the increase in risk varies from 1.5-3 fold[42, 45]. The exact mechanism linking BV and preterm birth is currently unclear. However, it has been suggested that BV associated organisms can ascend to the upper genital tract and initiate preterm labor via inflammatory processes at this site[40]. Studies of term labors have yielded a wealth of information about the processes which occur to induce the appropriate sequence of events, and these processes may be activated in an untimely manner in the setting of vaginal infection. Specifically, it is thought that bacterial PAMPs (Pathogen Associated Molecular Patterns) can activate proinflammatory signaling resulting in the production of IL-1β, TNF-α, IL-6, IL-8 and others. These cytokines can augment

their own production as well as result in the production of prostaglandins, matrix metalloproteinases, and recruitment of neutrophils and macrophages to the maternal fetal membranes, cervix, and myometrium[46]. Additionally, several studies have implicated elevated defensin levels during episodes of BV with an increased risk for preterm labor[42, 47]. Whether these defensins play an active part in initiating preterm labor, or are a marker of some other event (such as neutrophil influx) remains unclear. Figure 1.2 is a proposed model for how BV is linked to preterm labor. Interestingly, despite the association of increased proinflammatory cytokines, BV is not a state characterized by high proinflammatory cytokine production (IL-8, IL-6, and TNFα) in vaginal fluids[48-51], and attempts to treat the infection and limit the incidence of preterm labor have been highly unsuccessful[52-57]. This is suggestive of a state that precedes BV which is high in proinflammatory cytokines that is the main driver for BV associated preterm labor.

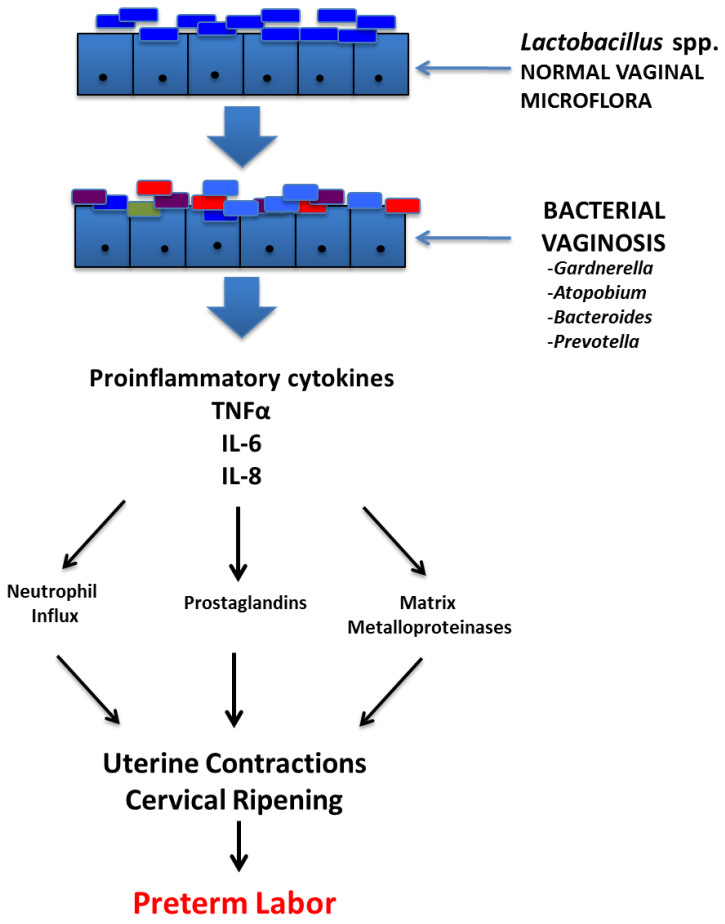


Fig 1.2 Proposed model for link between bacterial vaginosis and preterm birth. The link between preterm labor and bacterial vaginosis has been established epidemiologically, but no clear mechanism has been defined. It is suggested that in a BV state, the potential pathogens contribute to the production of proinflammatory mediators (IL-8, IL-6, and TNFα) which can then result in the production of prostaglandins as well as matrixmetalloproteinases. BV may be a marker for infection further up the female genital tract or inflammatory mediators produced locallly in the vagina may ascend to induce changes at the cervix and within the uterus.

1.3 Identification of a novel *Lactobacillus* species, *Lactobacillus iners*

Although the dominance of vaginal lactobacilli has been noted for over a century, culture independent techniques continue to expand our knowledge. Recently, the use of these

techniques expanded the repertoire of lactobacilli to include a novel species, *L. iners*[58]. This species has subsequently been shown to be a common constituent of the vaginal tract[59, 60], and in fact is the dominant species in approximately 30% of women[9]. Although ubiquitous in the vaginal tract, this organism was initially overlooked because the media used to isolate vaginal lactobacilli, de Man-Rogosa-Sharpe (MRS) agar[61], does not support the growth of *L. iners*. This inability to grow on media used to support most lactobacilli foreshadowed the unique and strange behavior of this organism in the vaginal tract.

1.3.1 Taxonomy and Physiology

Figure 1.3 shows a phylogenetic tree demonstrating the relatedness of *L. iners* to other lactobacilli. Like other lactobacilli, this organism is a Gram positive, facultatively anaerobic rod. Growth did not take place on MRS agar, and required blood agar. Interestingly, unlike other vaginal lactobacilli, this organism is noticeably shorter and fatter[58]. The genome of this organism is significantly reduced, being the smallest Lactobacillus genome to date with a single chromosome of approximately 1.3Mbp. Contained within the genome are several proteins which contribute to its ability to persist in the vaginal tract: several alkaline shock proteins as well as alternative sigma factors which aid in tolerating altered pHs as well as stress associated with infection. The genome also possesses iron-sulfur cluster assembly systems which are important for moving iron and sulfur to the appropriate locations within the cell as well as acting as sensors which can initiate responses to environmental changes[62].

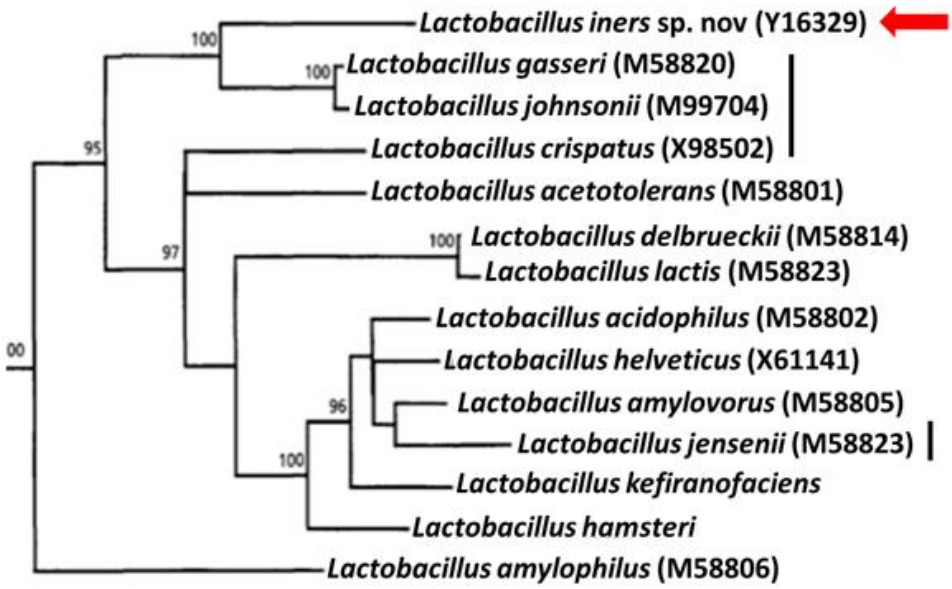


Fig 1.3 Unrooted tree showing the phylogenetic relationship between *Lactobacillus iners* and other *Lactobacillus* species. *L. iners* is indicated by an arrow. The most commonly isolated vaginal *Lactobacillus* species are indicated by a bar. Adapted from Falsen et al (1999)

1.3.2 Role in the Vaginal Tract

Although this organism is a member of the genus *Lactobacillus* and closely related to other vaginal lactobacilli, it seems to lack the protective effects traditionally ascribed to this group. Ravel et al. demonstrated that women with a microbiota dominated by this organism showed elevated and variable pH as well elevated and variable Nugent scores as compared to an *L. crispatus* dominated microbiota which had a lower and more homogenous pH and Nugent score profile. This large study suggests that a microbiota that is dominated by *L. iners* is less stable, and more prone to alterations of its composition, as would be seen with BV. It has also been suggested that *L. iners* represents a transitional species, present after treatment with

antibiotics[63] or after administration of exogenous estrogen[64]. Given that 70% of women who are treated for bacterial vaginosis will recur, the dominance of *L. iners* post treatment suggests that this organism contributes in some way to an imbalance in the vaginal tract. Several groups have taken this link one step further, to demonstrate an association between the presence of *L. iners* and the presence of other BV associated organisms[65]. This result is also consistent with other published work demonstrating that *L. iners* persists during episodes of BV, a condition in which *Lactobacillus* species are normally lost. While this does not necessarily link *L. iners* to causing changes in the vaginal microbiota, it is peculiar that this organism shows a positive association with BV associated microbiota. All of these data are further supported in a study by Srinivasan et. al, where it was demonstrated that the presence of *L. iners* is not associated with the absence of BV [36]. Another clear demonstration of the ability of *L. iners* to stimulate alterations of the microbiota was a study which examined the microbiota of pregnant women over time as a function of *Lactobacillus* status. In this cohort, they demonstrated that women with a vaginal micromicrobiota dominated by *L. iners* had a tenfold increased risk for conversion to an "abnormal vaginal micromicrobiota" as indicated by Gram stain[66]. While there is much to learn about the physiology of this organism and its role in the vagina, these data suggest that this organism does not adhere to the traditional view of vaginal lactobacilli as stabilizing, protective species.

1.4 Bacterial toxins

A wide variety of pathogenic organisms produce toxins. Broadly, toxins can be characterized as endotoxin and exotoxins. Endotoxins are a common feature of all Gram negative organisms,

and are non-proteinaceous components of the cell wall. The major endotoxin present in Gram negatives is Lipopolysaccharide (LPS), and is released as bacteria die and lyse. These endotoxins are able to activate the immune system via toll-like receptors (TLRs), resulting in cytokine release which, left uninhibited can result in fever, shock, and death. The other major classes of toxins are the exotoxins. These proteins are a group of proteinaceous toxins which are secreted by the organisms in response to a number of cues. In characterizing their activity, we can broadly classify exotoxins into three categories: those which act intracellularly (which includes cholera toxin and diphtheria toxin) and have enzymatic activity, those which have membrane damaging activity (including the CDCs), and those which are able to activate/stimulate the immune system inappropriately (pyrogenic exotoxins). These toxins play important roles in the pathogenesis of the organisms which produce them, conferring some sort of advantage over the host. The best known and studied toxin is diphtheria toxin, produced by *Corynebacterium diphtheria.* First discovered in 1888 by Yersin and Roux, this toxin is of the A/B type, which possess two components. For this group of toxins, the toxic activity is contained within the A subunit, and the B subunit is involved in binding to target cells and delivering the A subunit intracellularly. Diptheria toxin is secreted as a single polypeptide[67] and the B subunit interacts with heparin binding protein[68] which is then internalized via receptor mediated endocytosis. The A subunit possesses ADP-ribosyltransferase activity and transfers an ADP-ribose group on to elongation factor 2 (EF2), which results in inhibition of protein synthesis and ultimately localized cell death[69]. The membrane damaging toxins can destabilize membranes in a variety of ways: enzymatic activity against membrane lipids (exemplified by α-toxin of *C. perfringens* which has phospholipase C

activity), toxins with surfactant like activity allowing disruption of membranes (such as delta toxin of *S. aureus*)[70], and those which produce holes in cell membranes resulting in leakage of intracellular contents, influx of water, and ultimately cell lysis. This latter class, the pore forming toxins, are the largest class of bacterial toxins and are often important virulence factors[71]. These toxins are secreted as monomeric proteins, interact with target membranes via a membrane receptor, and then oligomerize to form the pore. This ability to diffuse within the lipid bilayer and oligomerize is the unifying feature of this class of toxins[72]. The PFTs can be categorized as α-PFTs and β-PFTs, based on the secondary structure utilized for membrane insertion. The α-PFTs insert amphipathic α helices into the membrane whereas β-PFTs insert an amphipatic β- barrel structure into the membrane[73]. For a more in-depth understanding of the α-PFTs, see the reviews cited here[74, 75]. The cholesterol dependent cytolysins are a major group within the larger family of β-PFTs and are discussed further below.

1.4.1 Regulation of toxin activity

Toxin activity is important for the pathogenesis of many organisms, conferring some advantage over the host. However, production of these bacterial toxins can be energetically costly, and often toxin production is required only at a particular time in the life cycle of the organism. Regulation of toxin activity can occur at the level of expression or post-translationally at the level of the function of the protein itself. Throughout the class of bacterial exotoxins, regulation can occur at both of these levels. Outlined here are just a few examples, to provide some context for studies carried out here examining regulation of CDC activity. As mentioned above, diphtheria toxin is one of the most widely studied toxins, and demonstrates regulation not only at the level of transcription but also at the level of activity of the protein itself.

Expression of diphtheria toxin is regulated by the availability of iron. When iron is abundant, the DtxR protein is expressed. This protein can then bind to regulatory sequences upstream of the *tox* gene encoding diphtheria toxin, preventing transcription[76, 77]. A similar mechanism has been noted for Shiga toxin as well as Shiga-like toxin[78]. Despite the sequencing of a number of CDC genes and their surrounding regions, no known regulatory sequences have been identified for these toxins. To date, no one has reported any environmental regulation of CDC expression, except for the CDC produced by *B. anthracis*[79]. At the level of actual protein function, pH can be an important regulator of toxin activity. This has been demonstrated for diphtheria toxin, where acidic pH has been shown to facilitate translocation of the toxin from the acidified endosome into the cytoplasm. Structural studies have demonstrated that upon exposure to acidic pH, conformational changes take place in the protein which "activate" the protein, exposing regions which will insert into the membrane[80]. The activity of some members of the CDC family (PFO and LLO) have been shown to be regulated by pH as well. The role that pH plays in regulating the activity of these CDCs is discussed further below. Overall, pH seems to be an important regulator of activity of bacterial toxins, and is investigated in the work presented here.

1.5 Cholesterol Dependent Cytolysins (CDCs)

The CDCs are a family of pore-forming toxins belonging to a larger class of β-barrel pore forming toxins. These β-PFTs are distinguished from the other major class of PFTs, the α-PFTs, by their use of amphipatic β-hairpins to create a β-barrel structure. The CDCs are distinguished within this larger group because of their absolute requirement for cholesterol in target

membranes, making them specific for eukaryotic cells. This unifying characteristic has been demonstrated in several studies as toxin activity can be inhibited by the addition of exogenous cholesterol[81] as well as by the depletion of membrane cholesterol[82]. Additionally, they are known to form extraordinarily large pores in target membranes, with diameters exceeding 15nm.

The events of pore formation and their molecular mechanisms have been extensively investigated over the past 50 years, and while a more complete picture has emerged, there is still much that is unknown about the pore formation and toxin activity. As noted before, there is an absolute requirement for cholesterol in target membranes, which affords these toxins a cellular receptor. Interestingly, a few toxins have been identified that require cholesterol for activity, but make use of an additional protein receptor to mediate initial membrane binding activity. These toxins, Vaginolysin (VLY) and Intermedilysin (ILY), and the revised role of cholesterol in pore formation will be discussed later. Upon membrane binding, an elegant and concerted series of events results in the oligomerization of monomeric toxins into a pore structure, leading to cell lysis.

The first CDCs were discovered more than a century ago [83] and as the number of cultivatable organisms grows and methods for looking at the genomes of cultivated and uncultivated organisms grows, we have greatly expanded the family to include 25 members produced by Gram positive organisms (Table 1.1). These toxins play crucial roles in the pathogenesis of diseases caused by these organisms and are required in various stages/roles in disease

progression. Initially thought to be a feature unique to Gram positive organisms, recent work has demonstrated the identification of two putative CDCs in two Gram negative organisms, desulfolysin (DLY) and enterolysin (ELY)[84]. Interestingly, unlike the Gram positive CDC producing organisms, for which the human body is a physiological niche, the Gram negative CDC producing organisms predominantly inhabit sediments and soils. This finding suggests roles in more than just disease pathogenesis, but also in protection against eukaryotic predators.

Whatever the role these toxins may play, a deeper understanding of these toxins and the mechanisms by which they make pore is critical to developing targeted therapies to combat these toxins. With the increasing emergence of antibiotic resistance and a better appreciation for the crucial protective role the normal micromicrobiota plays in health, it has become increasingly important to tailor therapies to these crucial virulence factors. These targeted therapies can disarm the offending organisms, while preserving the normal micromicrobiota and provide weaker selective pressure on the development of antimicrobial resistance.

Table 1.1 Family of Cholesterol Dependent Cytolysins (CDCs)

Bacterial genus	Species	CDC	Abbreviation
Gram Positive			
Arcanobacterium	*A. pyogenes*	Pyolysin	PLO
Bacillus	*B. alvei*	Alveolysin	ALY
	B. anthracis	Anthrolysin O	ALO
	B. cereus	Cereolysin O	CLY
	B. laterosporus	Latersporolysin	LSL
	B. thuringensis	Thuringiolysin O	TLO
Clostridium	*C. bifermentans*	Bifermentolysin	BFL
	C. botulinum	Botulinolysin	BLY
	C. chauvcei	Chauveolysin	CVL
	C. histolyticum	Histolyticolysin O	HTL
	C. novyi type A	Novyilysin O	NVL
	C. perfringens	Perfringolysin O	PFO
	C. sordellii	Sordellilysin	SDL
	C. septicum	Septicolysin O	SPL
	C. tetani	Tetanolysin	TLO
Gardnerella	*G. vaginalis*	Vaginolysin	VLY
Listeria	*L. ivanovii*	Ivanolysin	ILO
	L. monocytogenes	Listeriolysin O	LLO
	L. seeligeri	Seeligerolysin	LSO
Streptococcus	*S. canis*	Streptolysin O	SLO
	S. equisimilis	Streptolysin O	SLO
	S. intermedius	Intermedilysin	ILY
	S. pneumoniae	Pneumolysin	PLY
	S. pyogenes	Streptolysin O	SLO
	S. suis	Suilysin	SLY
Gram Negative			
Desulfobulbus	*D. propionicus*	Desulfolysin	DLY
Enterobacter	*E. lignolyticus*	Enterolysin	ELY

1.5.1 Monomer Structure

CDCs are secreted as water soluble monomers, and range in size from about 50-60kDa. These protein toxins all show high identity and similarity at the primary amino acid level, ranging from

40-70%[85]. Given this high degree of identity, it is likely that this entire family of toxins fold into similar three dimensional structures. Much of the structural data we have is based on PFO, and to date, only two of the members in this entire family have been crystallized, the other being ILY. Overall, CDC monomers have a four domain structure, which is discontinuous. Much work has been done in assigning various functions to each of these domains. While certain activities are localized to particular domains, it is very clear that individual domains are in communication with each other and pore formation requires concerted events between domains for functional pore formation. The most highly conserved portion of the protein is at the C-terminus, an 11 amino acid stretch known as the undecapeptide, or Trp-rich region. Figure 1.4 shows a comparison of the region in several members of this family. The role/function of this portion of the protein is discussed further below.

Figure 1.4. **Comparison of the undecapeptide region**. The undecapeptide or Trp rich region is highly conserved. Most CDCs posess the consensus sequence ECTGLAWEWWR. Several members demonstrate alterations of this sequence which may have functional consequences

1.5.2 Mechanism of pore formation

Fig 1.5 shows the overall process of pore formation for the cholesterol dependent cytolysins[86].

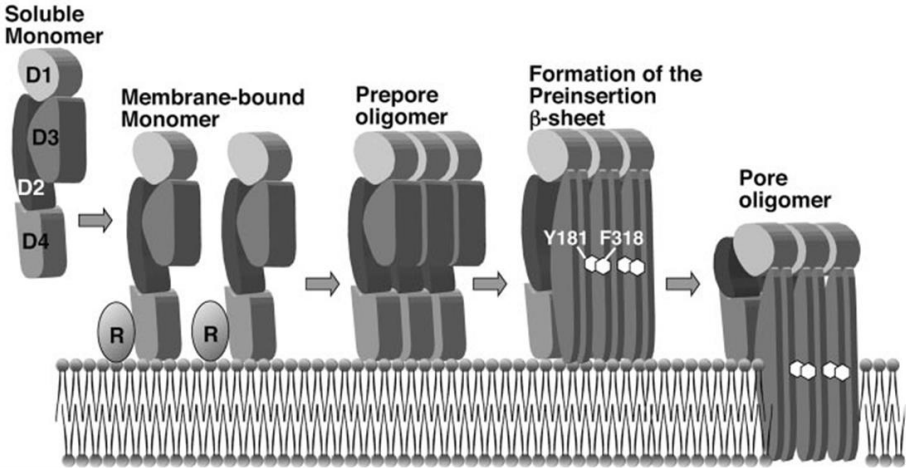


Fig 1.5. Model of CDC pore forming mechanism. Soluble monoomers interact with target membranes via a membrane receptor(R). In the case of the non-species restricted toxins, this receptor is membrane cholesterol and in the case of the species restricted toxins CD59 serves this purpose. Upon membrane binding, structural changes occur in domain 3 resulting in oligomerization of membrane bound monomers into a prepore structure. Further structural changes occur resulting in the unraveling of 3^{α} helical bundles to form TMH1 and TMH2. These TMHs from individual monomers insert into the membrane, forming a β barrel pore. Adapted from Tweten el al (2001).

1.4.2.1 Membrane Binding

As mentioned previously, for most of the CDCs (with the exception of ILY and VLY) cholesterol serves as the receptor for initial membrane interaction. Various pieces of evidence have implicated domain 4 in this membrane binding activity, and over the years the data has suggested different portions of this domain to be implicated in this activity, much of which was localized to the undecapeptide region. One group demonstrated modification of the conserved cysteine residue (in all CDCs except PLO and ILY) resulted in reduced membrane binding

activity[87]. It was later determined that this cysteine residue was not necessarily involved in membrane binding, but upon oxidation was then blocked by undefined molecules from the culture medium or from bacterial metabolism. Additional studies targeted the tryptophan residues present in the undecapeptide. The mutation of these residues initially seemed to alter membrane binding[88]. Additional studies revealed that rather than being involved in initial membrane binding to cholesterol in target membranes, these residues were important for initiating conformational changes in the protein that were important for pore formation. The actual portion of the protein which mediated cholesterol binding remained elusive until a study by Soltani et. al [89] demonstrated the importance of three hydrophobic loops L1-L3 in mediating cholesterol binding in target membranes. Further study into the mechanisms of membrane recognition then identified the cholesterol recognition motif (CRM) as a Threonine-Leucine pair in loop 1 which was found to be conserved in all known CDCs[90] . Figure 1.6 shows the conservation of this ThrLeu Pair in several CDCs.

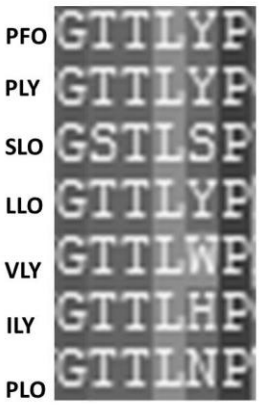


Figure 1.6 Comparison of the L1 loop in several known CDCs. The L1 loop is implicated in cholesterol binding. The Thr-Leu pair which is the determinant of cholesterol binding is indicated by the bar above it. This Thr-Leu pair is necessary for cholesterol binding activity and is present in all known CDCs.

1.4.2.2 Oligomerization

After membrane binding, the next step in the process of pore formation is oligomerization. For some time, it was unclear whether insertion of transmembrane helices in the protein occurred before or after oligomerization of monomeric toxin. After much examination, several groups using fluorescent probes and disulfide trapping [91] as well as modulating the temperature[92] confirmed the correct sequence of events. Upon membrane binding, via this Thr-Leu pair, membrane insertion of L2 and L3 as well as the undecapeptide occurs. It was initially thought that this was the region that then contributed to the final formation of the pore[93]. However, it was later shown that this region, although inserting did not embed itself deeply into the

membrane making it unlikely to be the portion which contributed to the final pore. This insertion event is crucial to pore formation, but because it then initiated further structural changes in the protein, not because it contributed anything to the ultimate pore structure. Specifically, the insertion of this protein then maintains the monomer in an orientation perpendicular to the membrane[94-96]. Additionally, insertion of the arginine residue present at the end of the undecapeptide is crucial for coupling membrane binding to the structural changes needed for the oligomerization process. Specifically, upon insertion of the undecapeptide, and this arginine residue, changes are transmitted to D3 of the protein, and specifically to the four β sheets that contribute to the final pore. The β4 sheet is normally not exposed due to hydrogen bonding to a short hydrophobic region termed the β5 loop. Upon membrane binding, insertion of a tryptophan in domain 3 into the membrane initiates the movement of the β5 loop away from the β4 sheet by virtue of a flexible glycine linker[97]. This exposes β4 which then allows it to interact with the exposed surface of the β1 sheet allowing for oligomerization into a prepore complex[98]. Figure 1.7 shows the proposed mechanism for these structural changes[99]. This prepore however must undergo further structural changes to form the final pore structure.

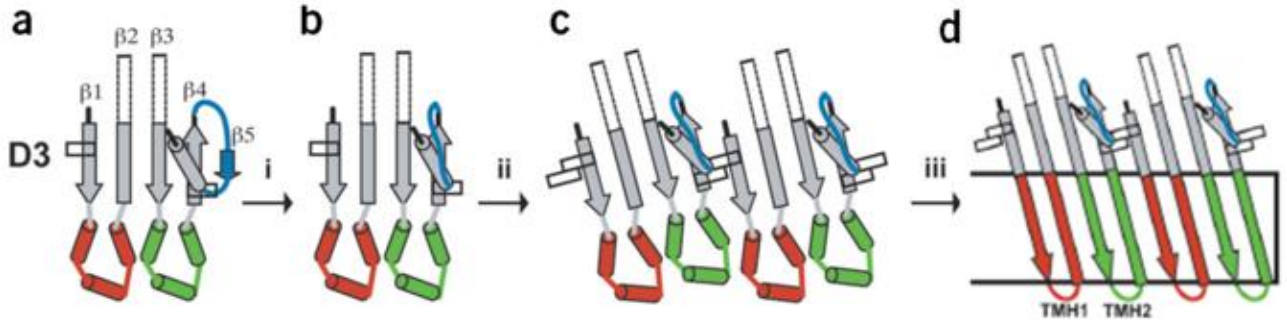


Figure 1.7 Membrane binding initiates conformational changes promoting oligomerization. Monomeric toxin binds to the surface of the target cell via cholesterol mediated by domain 4. This initiates several structural changes which are transmitted to domain3. Specifically, the β5 loop rotates away from the β4 sheet, uncovering the oligomerization interface, and allowing the β4 sheet of one monomer to intearct and oligomerize with the β1 sheet of an adjacent monomer. Adapted from Ramachandran et. al (2004)

1.4.2.3 Pore Formation

The rate limiting step for pore formation is the formation of this prepore complex. Studies using disulfide locked mutants demonstrated that toxins could oligomerize on the surface, and upon reduction of the disulfide, pore formation proceeded with faster kinetics then if these toxins were pre-reduced in solution before being incubated with membranes[91]. The interaction between adjacent monomers is crucial to the final steps of pore formation: the disruption of the domain 2 and domain 3 interface, and insertion of the transmembrane helices (TMHs) present in domain 3. The events between oligomerization and insertion of TMHs have not been completely worked out, but it is clear that monomers must align their transmembrane helices and in a concerted manner insert these TMHs to produce the final pore[100].

1.5.3 Properties of Specific Cholesterol Dependent Cytolysins

Despite the high level of sequence similarity/identity and the seemingly similar mechanisms of action, further study of these toxins has demonstrated the evolution of specific and unique activities to the members of this family. The unique characteristics of a few of these toxins are outlined below.

1.5.3.1 Listerolysin O (LLO)

Listeriolysin O (LLO) is the cholesterol dependent cytolysin produced by *L. monocytogenes*. This organism is particularly interesting as it is one of the few CDC producing organisms known to have an intracellular lifestyle. Various studies have demonstrated the importance of this pore forming toxin in phagosomal escape[101, 102], and intracellular survival and replication. This toxin has evolved specific mechanisms making it perfectly suited to its intracellular lifestyle, operating maximally at acidic pH and rapidly losing activity at neutral pH, as would be experienced upon escape from the acidified phagosome and into the more neutral cytosol. Extensive investigation has led to the identification of a triad of acidic residues in domain 3 of the protein. This triad of charged residues acts a sensor, detecting an increase in pH and causing a rapid and irreversible unfolding of the protein and loss of activity[103]. The importance of this pH dependent activity has been shown to be crucial to the lifestyle of this organism, as replacement with the pH "insensitive" toxin, PFO, completely inhibited the growth and propagation of this organism[101].

1.5.3.2 Perfringolysin O (PFO)

Perfringolysin O is perhaps one of the most studied CDC and is the basis for many of the structural studies that have been carried out to date. Given that it is one of two toxins in this family which have been crystallized and for which the structure has been solved, it forms the logical basis for a number of studies examining structural aspects of the pore forming mechanism. Recently, this organism was shown to possess an intracellular component to its lifestyle[104]. As with LLO, this toxin has also evolved mechanisms making it well suited to its role in the acidified phagosome, showing enhanced activity at low pH. Unlike LLO, this toxin maintains hemolytic activity at more neutral pH, and to date the mechanism for this enhancement at low pH has not been elucidated. It has been suggested that the enhancement of activity at low pH is due to a partial unfolding event of the TMHs that primes the protein for membrane insertion[105].

1.5.3.3 Vaginolysin (VLY)

VLY is the CDC produced by the vaginal bacterium (and potential pathogen) *G. vaginalis*. It is hypothesized that this organism (and its toxin) plays a role in the pathogenesis of bacterial infections, specifically in the etiology of bacterial vaginosis (BV). This toxin was initially identified in crude extracts of bacterial preparations of *G. vaginalis*. Despite possessing characteristics comparable to that of PFO, this toxin was initially thought not to be a part of the CDC superfamily as it was inactivated by β-mercaptoethanol[106]. It was later discovered, after genomic data became available and recombinant protein was produced, that the basis for this was the absence of the cysteine residue in the undecapeptide region. This toxin, as well as

Intermedilysin produced by *Streptotoccus intermedius*, represents the species specific members of the cholesterol dependent cytolysin superfamily, showing a restriction for human derived targets[26]. The basis for this was discovered to be an alteration in the molecule that mediated initial interaction with target membranes. While it is accepted that the non-species specific toxins utilize cholesterol to mediate initial membrane interactions, the human specific toxins, VLY and ILY, utilize the GPI linked protein CD59 to mediate membrane recognition. Interestingly, despite the initial interaction being mediated by this protein receptor, cholesterol is absolutely crucial to the activity of the protein, and it has been shown that the Thr-Leu pair which mediates cholesterol recognition is conserved in these species specific toxins.

1.5.3.4 Streptolysin O

Streptolysin O is produced by several members of the genus *Streptococcus*. This toxin possesses several of the features common to the non-species specific CDCs, including an invariant undecapeptide sequence (ECTGLAWEWWR) and the conserved Thr-Leu pair which mediates cholesterol binding. It has been most extensively studied in the context of *S. pyogenes* infection, which is mainly associated with wound infections. This toxin is the largest member of this family, on the basis of primary amino acid sequence, with 571 residues. This toxin was crucial in suggesting that CDCs do more than just punch holes in target membranes and cause cell lysis. It was discovered that SLO, after pore formation translocated the bacterial protein NADH-glycohydrolase into the target cells, and this required an N-terminal region that was missing in all other CDCs[107, 108]. To date, translocation of other factors or translocation

by other toxins has not yet been demonstrated, although it has been shown that the pore can facilitate the translocation of peptidoglycan (but probably not in a specific manner)[109]

1.5.3.5 Pneumolysin

Pneumolysin (PLY) is produced by the Gram positive organism *Streptococcus pneumoniae.* This organism is responsible for a number of different diseases including pneumonia, meningitis, and otitis media. The importance of this virulence factor to disease pathogenesis has been highlighted in several studies[110-113]. Unlike other members of the cholesterol dependent cytolysin family, this toxin lacks the N-terminal secretion signal. PLY is seen to accumulate in the cytoplasm of the bacterium and is released upon autolysis of the cell. PLY is also unique in its ability to activate complement[114]. Interestingly, unlike many other toxins, PLY can form oligomeric structures in solution with no exogenous membranes or cholesterol. The importance of this activity in disease pathogenesis is not yet clear.

1.6 Statement of Hypothesis

Lactobacillus iners is a newly identified constituent of the vaginal tract. Lactobacillus species are thought to provide a protective effect within the vaginal tract, maintaining proper pH and preventing overgrowth by potentially pathogenic species. Epidemiological data suggests that *Lactobacillus iners* rather than contributing to stability of the vaginal tract may actually contribute to alterations of the vaginal microbiota (as seen in BV). Furthermore,

attempts to treat BV and limit the adverse outcomes associated with it have been highly unsuccessful. Given *L. iners* ability to persist in both BV and non-BV states, as well as its association with increased incidence of BV, we speculate that *L. iners* has pathogenic potential and can contribute to BV pathogenesis and BV associated outcomes. We sought to identify and characterize virulence factors produced by *L. iners* and understand their role in BV and BV associated preterm labor. Specifically we,

1. Identified and characterized a putative pore forming toxin produced by *Lactobacillus iners* described in Chapter 2
2. Investigate the mechanism by which activity of this toxin is regulated described in Chapter 3
3. Characterize the responses of the vaginal epithelium to the bacteria and its toxin described in Chapter 4

Chapter 2: Identification and Characterization of Inerolysin, the Cholesterol Dependent Cytolysin produced by *Lactobacillus iners*

Adapted from Rampersaud et. al (2010)

2.1 Introduction

The cholesterol-dependent cytolysins (CDCs) are a family of protein toxins produced by a wide range of Gram-positive bacteria. CDCs share several characteristics, including a four- domain structure, a requirement for membrane cholesterol for efficient activity, and an ability to form large pores in host cells, exceeding 150 Å in diameter[86]. In general, soluble CDC monomers are secreted into the extracellular environment and bind to target cell membranes through direct recognition of cholesterol or, in the cases of the human-specific toxins vaginolysin (VLY) from *Gardnerella vaginalis* and intermedilysin (ILY) from *Streptococcus intermedius*, via recognition of human CD59 on the target cell surface[26, 115]. Following membrane association, CDCs oligomerize to form a prepore structure, a process that is dependent upon the availability of cholesterol [89, 99, 115, 116]. In many cases, CDCs are required for virulence for their cognate organisms, and rather than acting solely as cytolytic toxins, CDCs may have more sophisticated roles in disease pathogenesis [102, 117, 118]. Understanding CDC evolution and host specificity is of considerable interest and has been limited by incomplete knowledge of the diversity of the CDC family. In particular, characterization of cytolysins most closely related to those in which host specificity has evolved may provide additional insights into the mechanism and effects of such restriction. *Lactobacillus iners* is a relatively recently recognized member of the human vaginal microbiota[58-60] that was initially overlooked because of its

inability to grow on de Man- Rogosa-Sharpe agar, which is normally used to isolate vaginal lactobacilli. In healthy women, the vaginal microbiota is dominated by *Lactobacillus* species, *with L. crispatus, L. gasseri, and L. jensenii* being the most commonly cultivated[119]. These organisms are thought to play a role in resistance of the vaginal tract to colonization by pathogens, possibly through the production of lactic acid. *L. iners* is unusual among lactobacilli in that it may be detected during bacterial vaginosis (BV), a state in which *G. vaginalis* generally predominates and other Lactobacillus species are only rarely found at the vaginal mucosal surface[63, 66, 120, 121]. More recently, culture-independent studies of the vaginal microbiota have demonstrated that *L. iners* vaginal colonization may be considerably more prevalent than previously recognized, and in some cases it may be the most abundant organism detected[122, 123]. Other sequenced Lactobacillus strains lack identifiable CDC genes. Given the unusual biology of *L. iners* and its similarities to *G. vaginalis*, we performed a bioinformatic search for genes that might encode a CDC in the *L. iners* genome. Here we report the identification, cloning, and characterization of inerolysin (INY), the *L. iners* CDC. The description of INY expands the CDC family to include a nonspecific toxin with the greatest sequence similarity to VLY and ILY, the two species- specific members of the CDC family. Further study of this newly identified CDC will increase our understanding of the evolution of the CDC family and the role of *L. iners* in vaginal physiology.

2.2 Materials and Methods

2.2.1 Bacterial strains and cell lines

L. iners strains were grown on human blood bilayer-Tween agar. *L. iners* type strain DSM 13335, the genome of which has been sequenced, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. L. iners clinical isolates CCUG38955A, CCUG44023, CCUG44137, CCUG44284, CCUG46933, CCUG28746, CCUG32387, CCUG24626, and CCUG35443B were obtained from the Culture Collection of the University of Göteborg. *L. iners* strain ATCC 55195 was obtained from the American Type Culture Collection. Eukaryotic cell lines HeLa (ATCC CCL-2) and COS-7 (CRL-1651) were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM sodium pyruvate, and 10 g/ml ciprofloxacin.

2.2.2 Cloning and expression of CDCs

The open reading frame (ORF) containing INY lacking its predicted signal sequence was amplified by PCR from L. iners DSM 13335 genomic DNA using primers NheI-INY-F (GCCGCCGCTAGCA ATACTGAGCCAAAAACAGCTATTG) and XhoI-INY-R (GCCGCCCTCG AGTTAGTCATTTTTTACTTCTTCTTTG; restriction sites are underlined). The pneumolysin (PLY) ORF was amplified from Streptococcus pneumoniae strain D39 genomic DNA using primers NdeI-PLY-F (GCCGCCC ATATGGCAAATAAAGCAGTAAATGAC) and PLY-R-XhoI (GCCGCCCT

CGAGCTAGTCATTTTCTACCTTATCTTC). The products were cloned via the indicated restriction sites into the pET28a vector (Novagen) in order to generate N-terminally hexahistidine- tagged constructs, verified by sequencing, and transformed into T7 Express Iq competent E. coli (New England BioLabs). Expression strains were grown in LB with 50 g/ml kanamycin for 5 h, and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4.5 h. Bacterial cells were pelleted by centrifugation and lysed in a buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) containing a protease inhibitor cocktail and Benzonase nuclease. Lysates were cleared by centrifugation, and supernatants containing His-tagged proteins were purified with Ni-nitrilotriacetic acid agarose beads (Qiagen) according to the manufacturer's instructions. Purified proteins were dialyzed against lipopolysaccharide-free phosphate-buffered saline (PBS) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ overnight at 4°C, and protein concentrations were determined by a modified Bradford assay (Bio-Rad).

2.2.3 Detection of the INY gene in isolates of *L. iners*

The presence of the gene for INY in several clinical isolates of L. iners was detected by PCR from genomic DNA using primers INY-test-F (CAGCAACACCTGGGTTAGAACTATC) and INY-test-R (CAGGTGCTCTTTTCAAGGCAGAC), targeting an internal region of the INY ORF. Amplification was carried out using Taq DNA polymerase (NEB).

2.2.4 Bioinformatic analysis

Protein sequence prediction and alignment were carried out using MacVector software (version 11; MacVector Inc.). Other sequences were obtained from the GenBank/Entrez Protein database (National Center for Biotechnology Information [NCBI]). N-terminal signal sequences were predicted with SignalP (version 3.0, available at http://www.cbs.dtu.dk /services/SignalP/)[124]. Alignments for phylogenetic analysis were done in BioEdit v7.04 (Ibis Therapeutics) using ClustalW for amino acids and then toggled back to nucleotides for further analysis.

2.2.5 Phylogenetic analyses

Phylogenetic analyses were performed using three methods: neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). The optimal trees obtained by all of these techniques were almost identical. NJ analysis was done with PAUP[125] by using the minimum evolution criterion, allowing branch length to be negative except when calculating tree scores (for which they were set to zero) and breaking ties randomly. MP analyses were also done in PAUP using 1,000 random addition (RA) steps, followed by tree branch reconnection (TBR) swapping using the Multrees option in PAUP. Gaps were treated as a state, and all characters and state transformations were weighted equally. Bootstrap values were calculated using 100 bootstrap iterations using 100 replicates of RA, followed by TBR, in each iteration. ML analyses were performed with RAxML Blackbox (http://phylobench.vital-it.ch/raxml-bb/index .php)

using a GTRGamma I model. Node support was assessed with 100 rapid bootstrap replicates using RAxML.

2.2.6 INY Western blotting

The indicated *L. iners* strains were grown in Columbia broth with 5% defibrinated sheep blood and 10% fetal bovine serum for 2 days at 37°C in 5%CO_2. Bacterial cells were pelleted by centrifugation and lysed with BugBuster Protein Extraction Reagent (Novagen) containing Benzonase nuclease. Supernatants were concentrated from 5 ml to 500 µl using an Amicon 10-kDa centrifugal filtration device (Millipore). Supernatants were run on a 4 to 12% gradient polyacrylamide gel (Invitrogen). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% milk, and probed with one of two murine anti-PLY monoclonal antibodies as indicated below (Santa Cruz Biotechnology, 1:1,000 dilution, or Novacastra, 1:250,000 dilution). The primary antibody was detected with horseradish peroxidase-conjugated anti- mouse IgG by enhanced chemiluminescence.

2.2.7 Live-cell imaging of phalloidin entry into epithelial cells

HeLa cells were grown to confluence on uncoated glass bottom culture dishes (MatTek) and washed in PBS with 1 mM CaCl2 and 1 mM MgCl2. Phalloidin-Alexa Fluor 568 (Invitrogen) was added to a final concentration of 3.3 nM, and the cells were treated with INY (final

concentration, 1.8 μg/ml) or a vehicle control. Images were acquired on a Zeiss Axio Observer inverted microscope with appropriate filters every 30 s.

2.2.8 Erythrocyte lysis assays

The use of human erythrocytes from healthy adult volunteers after verbal informed consent was obtained was approved by the Columbia University Institutional Review Board (protocol IRB-AAAC5641). Defibrinated sheep and horse blood was obtained from Fisher Scientific. Erythrocytes were washed in sterile PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. For endpoint assays, 100 μl of a 1% washed erythrocyte solution was mixed with 100 μl of toxin in a 96-well V bottom plate and incubated for 30 min at 37°C and 5% CO_2. After 30 min, the plates were spun at 2,000 rpm to pellet erythrocytes. Supernatant was removed, and the optical density at 415 nm (OD415) was measured. In the indicated experiments, the toxin was incubated with cholesterol dissolved in chloroform or with chloroform alone (vehicle control) for 10 min at room temperature before use. In some experiments, the assay was carried out in the presence of dithiothreitol (DTT) or a vehicle control. In the indicated experiments, toxin was incubated with polyclonal anti-VLY rabbit serum or preimmune control rabbit serum (25) on a rotary shaker for 20 min at 4°C. For the kinetic assay, absorbance at 700 nm was measured every minute with an Infinite 200 microplate reader (Tecan).

2.2.9 LDH assay

HeLa cells were grown to confluence in a 24-well plate and weaned from serum and antibiotics overnight. Cells were incubated with toxin diluted in MEM for the indicated time periods. A 150-□l volume of supernatant was used to assay lactate dehydrogenase (LDH) release with the Cytotoxicity Detection Kit (Roche). The 100% lysis control was an identical well of HeLa cells concurrently treated with 1% Triton X-100 in MEM.

2.2.10 Epithelial p38 MAPK phosphorylation

HeLa cells were grown to confluence in a 24-well plate and weaned from serum and antibiotics overnight. Cells were incubated with CDCs diluted in MEM for the indicated time periods. Cells were lysed in radioimmunoprecipitation assay buffer (20 mm Tris [pH 7.4], 137 mM NaCl, 10% glycerol, 2 mm EDTA [pH 8.0], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) with protease inhibitors and phosphatase inhibitor cocktail (Sigma), separated on a 4 to 12% polyacrylamide gel, and transferred to a PVDF membrane. Membranes were blocked in 5% milk in Tris-buffered saline–Tween 20, probed with phospho-p38 mitogen-activated protein kinase (MAPK) antibody (Cell Signaling; 1:1,000), and detected with anti-rabbit IgG (1:5,000) by enhanced chemiluminescence. Blots were stripped in 4 N NaOH for 30 min, reblocked in 5% milk, and reprobed using anti-p38 MAPK antibody (Cell Signaling; 1:1,000).

2.2.11 Statistical analyses

Hemolysis assay results are expressed as average values from six readings from one representative experiment. Each experiment was repeated three times. Data were analyzed by one-way analysis of variance, followed by Tukey's posttest. For the antibody protection assay, the data shown are from one representative experiment. Data were analyzed by Student t test. A P value of 0.05 was considered significant. All statistical analyses were carried out using Prism 4 software (GraphPad)

2.2.12 Nucleotide sequence accession numbers

The accession number for INY from *L. iners* DSM 13335 is ZP_05744302 (annotated as perfringolysin O). Protein sequence data for PLY from *S. pneumoniae* TIGR4, R6, and Taiwan19F-14 are available from the NCBI under accession numbers AAK75991, AAL00542.1, and ACO23064.1, respectively. Other NCBI accession numbers are as follows: VLY from *G. vaginalis* ATCC 14019 and ATCC 14018, ACD39460 and ACD39459, respectively; ILY from *S. intermedius* UNS38 and UNS46, BAE16324 and BAA89790, respectively; LLO from *L. monocytogenes* NICBP 54006, ACF40759; ivanolysin (IVN) from *Listeria ivanovii* 16328, P31831; anthrolysin O (ALO) from *Bacillus anthracis* A0248, YP_002867524; cereolysin (CER) from *Bacillus cereus* B16, AAX88798; mitilysin (MLY) from *Streptococcus mitis R5II*, ABK58696; suilysin (SLY) from *Streptococcus suis 3*, CAC94852; perfringolysin O (PFO) from *Clostridium perfringens 13*, NP_561079; alveolysin (ALV) from *Paenibacillus alvei,* AAA22224; pyolysin (PYO)

from *Arcanobacterium pyogenes* BBR1, AAC45754. Corresponding nucleotide sequences were obtained from records linked to these amino acid sequences.

2.3 Results

2.3.1 The genome of *L. iners* contains a putative CDC

A basic local alignment search of the draft *L. iners* DSM 13335 genome sequence revealed an ORF with 51.9% identity and 68.4% similarity to VLY, the *G. vaginalis* CDC. This ORF was not found in available genome sequences from other *Lactobacillus* species (data not shown). The ORF contained a predicted signal sequence with a predicted cleavage site between amino acids 31 and 32. Primers were created to amplify a region in the ORF from DSM 13335 and were subsequently used to detect the presence of this putative CDC in the genomes of several other clinical isolates of *L. iners* (Fig 2.1A). A band of approximately 700 bp was detected after PCR of each of the 11 *L. iners* strains tested, indicating that the presence of this putative toxin is common to this species and not solely a feature of DSM 13335. The predicted amino acid sequence of INY exhibits sequence similarity and identity with diverse CDC family members (Table 1), with the greatest similarity to ILY and VLY, the human-specific CDCs. The nucleotide sequences of INY were used to generate phylogenetic trees by the NJ, MP, and ML methods, all of which displayed essentially the same topology (Fig 2.1B). These trees demonstrated that the putative CDC produced by *L. iners* falls into the Streptococcus CDC group including PLY and ILY and is more distantly related to CDCs from Listeria, Clostridium, and Bacillus. All 11 of the INY sequences were on a single branch with bootstrap support of 100%.The predicted INY ORF from

L. iners DSM 13335 was cloned into the pET28a expression vector in order to generate an N-terminal hexahistidine fusion, and recombinant INY (rINY) protein was purified. SDS-polyacrylamide gel electrophoresis analysis revealed a protein with a size of approximately 55 kDa (Fig 2.2A). By Western blotting, INY was detected using a monoclonal antibody directed against PLY (Fig 2.2B), as well as a polyclonal antibody against VLY (data not shown). In order to determine whether INY was produced and secreted, we assayed the culture supernatants of several *L. iners* strains for the presence of this putative CDC. A band consistent with the size of processed (lacking the signal sequence) INY was detected in the supernatants of all of the *L. iners* strains but not in the culture supernatant of *E. coli* (Fig 2.2C). Additionally, a higher-molecular-weight band was detected in *L. iners* supernatants which may represent either unprocessed INY released upon bacterial lysis or INY that has undergone an unclear type of posttranslational modification. There was variation among the *L. iners* strains with respect to the amount of INY produced (Fig 2.2C)

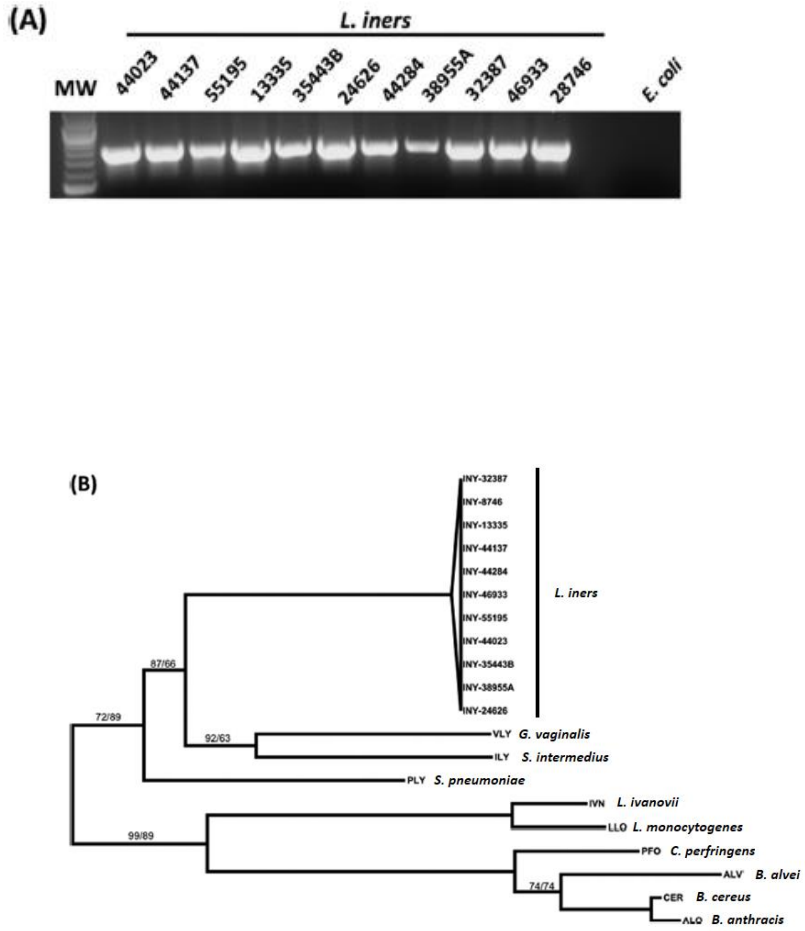


Figure 2.1. The genome of *Lactobacillus iners* contains an ORF encoding a putative CDC. (A) Amplification of the putative cholesterol dependent cytolysin from several strains of *Lactobacillus iners*. Primers were created to detect an internal region of INY. MW represents the DNA ladder. (B) Panel B shows the unrooted phylogeny. The topology shown was obtained independently using maximum parsimony (MP), maximum likelihood (ML) and neighbor-joining techniques (see methods). Numbers on branches are bootstrap percentages for MP and ML techniques (MP/ML). All bootstrap percentages are 100/100 unless otherwise indicated.

Table 2.1 Similarities and Identities of the primary amino acid sequences of INY and other CDCs

CDC	% Similarity	% Identity
ILY	69.2	48.8
VLY	68.4	51.7
PLY	67.1	48.9
MLY	62.3	46.0
SLY	62.3	46.0
IVN	59.6	39.1
ALV	58.6	37.8
ALO	57.4	36.5
CER	57.2	36.9
PFO	57.1	37.7
PYO	54.7	38.6

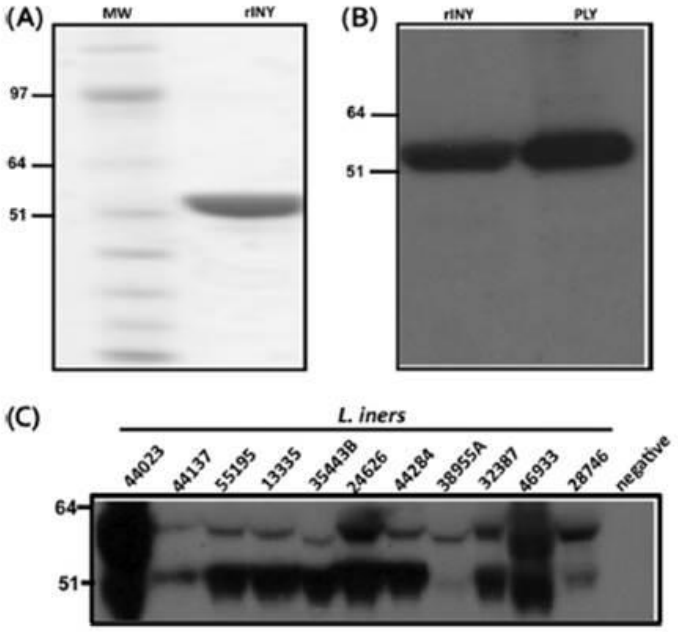


Figure 2.2 The putative cholesterol dependent cytolysin, inerolysin, resembles other cholesterol dependent cytolysins. (A) Purification of an approximately 57kDa protein (rINY, recombinant INY) from *E. coli* strain ARE173. **(B)** rINY and PLY probed with monoclonal anti-PLY which recognizes the undecapepetide reigon at the C-terminus of the protein**. (C)** Western blot of the concentrated supernatant from growth of several clincial isolates of *L. iners*. INY was detected using monoclonal PLY antibody. Concentrated *E. coli* supernatant was included as a negative control. Purified recombinant INY was included as a positive control.

2.3.2 INY is a functional broad-host-range cytolysin and is inhibited by anti-VLY antibody

rINY lysed murine, ovine, and human erythrocytes in a dose-dependent manner (Fig 2.3A). This lack of species specificity was in contrast to the previously described human specificity of ILY (Fig 2.3B) and was consistent with the behavior of PLY from *Streptococcus pneumoniae* (Fig 2.3C). Polyclonal anti-VLY antibody protected ovine erythrocytes from INY-mediated lysis in a dose-dependent manner (Fig 2.3D).

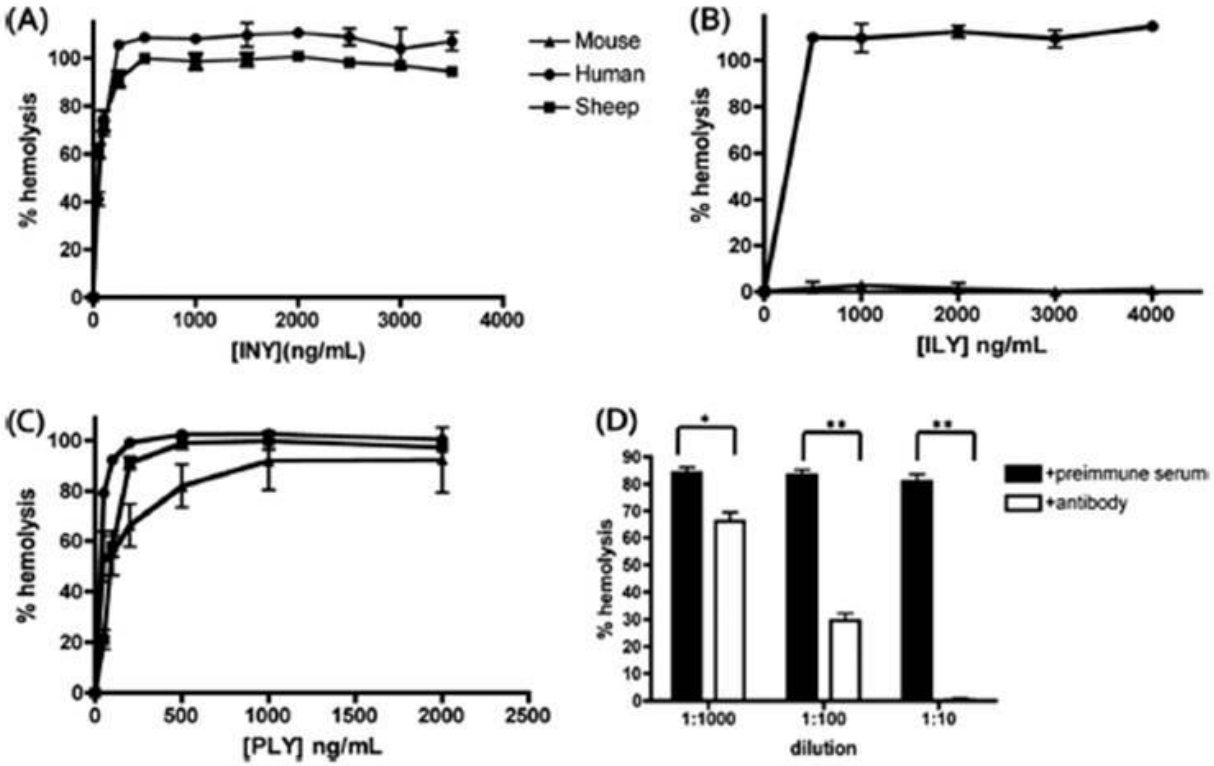


Figure 2.3 Recombinant INY shows non-species specific hemolytic activity consistent with other cholesterol dependent cytolysins. (A) INY incubated with sheep, mouse, and human erythrocytes for 30 min at 37°C/5%CO_2. For all hemolysis assays, intact erythrocytes were pelleted and hemoglobin release measured by OD_{415} of the supernatant. **(B)** ILY incubated with sheep, mouse, and human erythrocytes for 30 min **(C)** PLY incubated with sheep, mouse, and human erythrocytes for 30 min **(D)** INY (1.5 µg/mL) incubated with sheep erythrocytes for 30 min at 37°C/5%CO_2 in the presence of varying concentrations of anti-VLY antibody and corresponding dilutions of preimmune serum.

2.3.3 Cholesterol inhibits INY activity

CDCs bind cholesterol through a mechanism dependent on a Thr-Leu pair located in CDC domain 4 (10). This Thr-Leu pair is conserved in INY (Thr-507 and Leu-508 in INY from strain DSM 13335). The presence of excess cholesterol inactivates CDCs (3, 12, 13, 19, 20, 32).

Exogenous cholesterol inhibited INY-mediated lysis of ovine erythrocytes in both endpoint and kinetic assays (Fig 2.4)

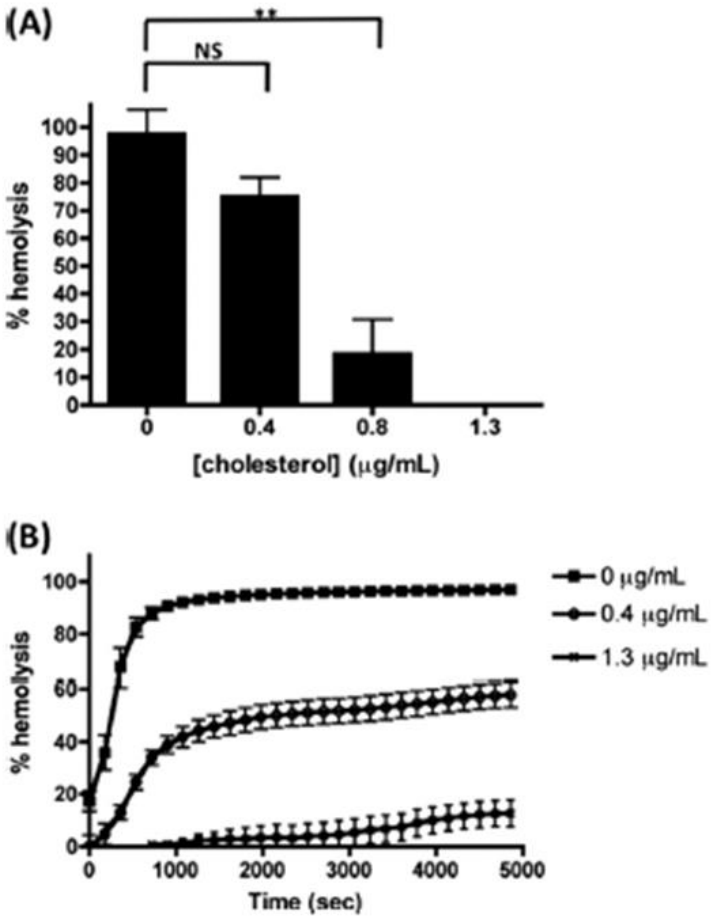


Figure 2.4 Recombinant INY shows cholesterol dependent hemolytic activity. (A) Recombinant INY (1.25 μg/mL) was incubated with sheep erythrocytes in the presence of indicated concentrations of cholesterol or vehicle control. Hemoglobin release was measured by OD_{415} of the supernatant after 45 minutes. *P* values for this and subsequent figures are as follows: *<0.05, **<0.001, ***<0.0001 **(B)** Recombinant INY (1.25 μg/mL) was incubated with sheep erythrocytes in the presence of indicated concentrations of cholesterol or vehicle control. Hemolysis was measured using OD_{700} every 3 minutes for a total of 1 hour.

2.3.4 INY activity is enhanced by DTT

CDCs were formerly known as “thiol-activated cytolysins,” which denoted the increase in lytic activity seen in many CDCs in the presence of reducing agents[126-130]. Thiol activation is dependent on the sequence of the undecapeptide, a highly conserved 11-amino-acid sequence in domain 4. The consensus undecapeptide, ECTGLAWEWWR, has a cysteine residue in the second position. This cysteine residue must be maintained in a reduced state for full toxin activity. Both ILY and VLY have unusual undecapeptides that lack this cysteine. However, the predicted primary amino acid sequence of INY contains the consensus undecapeptide. Thus, we predicted that INY would be thiol activated. Addition of DTT to a concentration of INY (200 ng/ml) that brought about low-level (30%) lysis alone led to a modest increase in the efficiency of lysis (Fig 2.5)

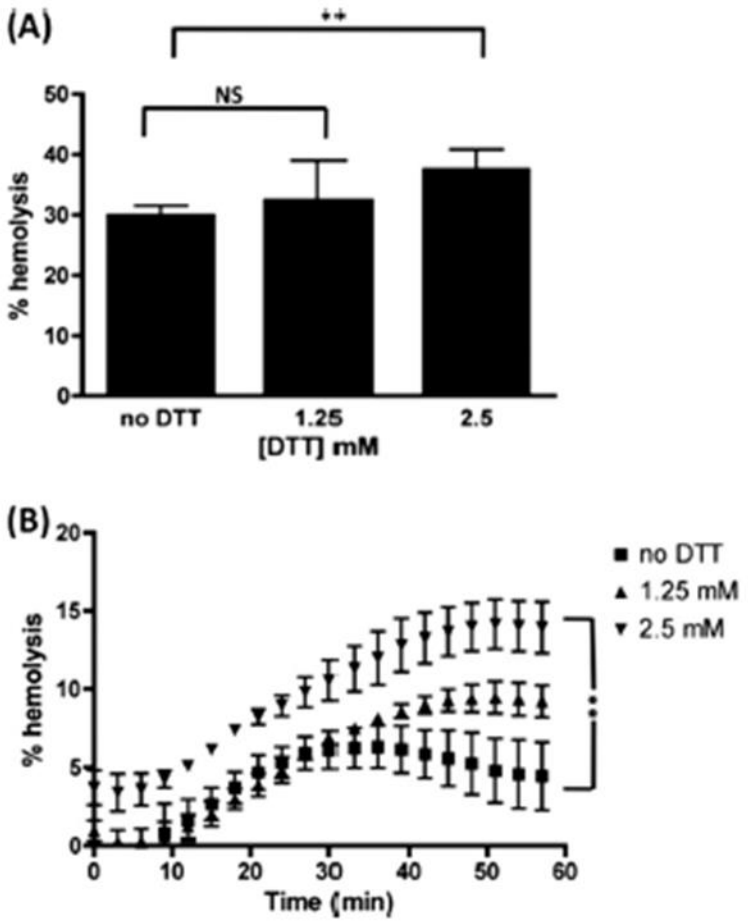


Figure 2.5 Recombinant INY is a thiol-activated cytolysin. (A) INY (200 ng/mL) was incubated in the presence of varying concentrations of DTT or vehicle control for 5 minutes at room temperature. This toxin was then used in a hemolysis assay to assess pore-formation. Hemoglobin release was measured by OD_{415} of supernatant after 45 minutes. **(B)** 200 ng/mL of toxin was treated as above. Hemoglobin release was measured by OD_{700} of supernatant every 3 minutes for 60 minutes

2.3.5 INY forms functional pores in epithelial cells and activates proinflammatory signaling.

L. iners colonizes the vaginal epithelium, and epithelial cells may represent a more physiologically relevant target for INY than erythrocytes. INY lysed human cervical epithelial (HeLa) cells in a dose-dependent manner, albeit at higher concentrations than those required for hemolysis (Fig 2.6A). This effect was not restricted to HeLa cells, as both COS-7 (Fig 2.6B)

and A549 (data not shown) cells were susceptible to INY-mediated lysis. Epithelial cells detect sublytic concentrations of pore-forming toxins and initiate p38 MAPK signaling through a mechanism dependent on sensation of osmotic stress (27). Consistent with the activity of other pore-forming toxins, INY activated p38 MAPK at sublytic concentrations (3 μg/ml) in both HeLa (Fig 2.6C) and COS-7 (Fig 2.6D) cells. Phalloidin, which is normally restricted from the interior of the cell, gains access to the cytoplasm of HeLa cells following treatment with INY (Fig 2.6E), consistent with the formation of functional pores.

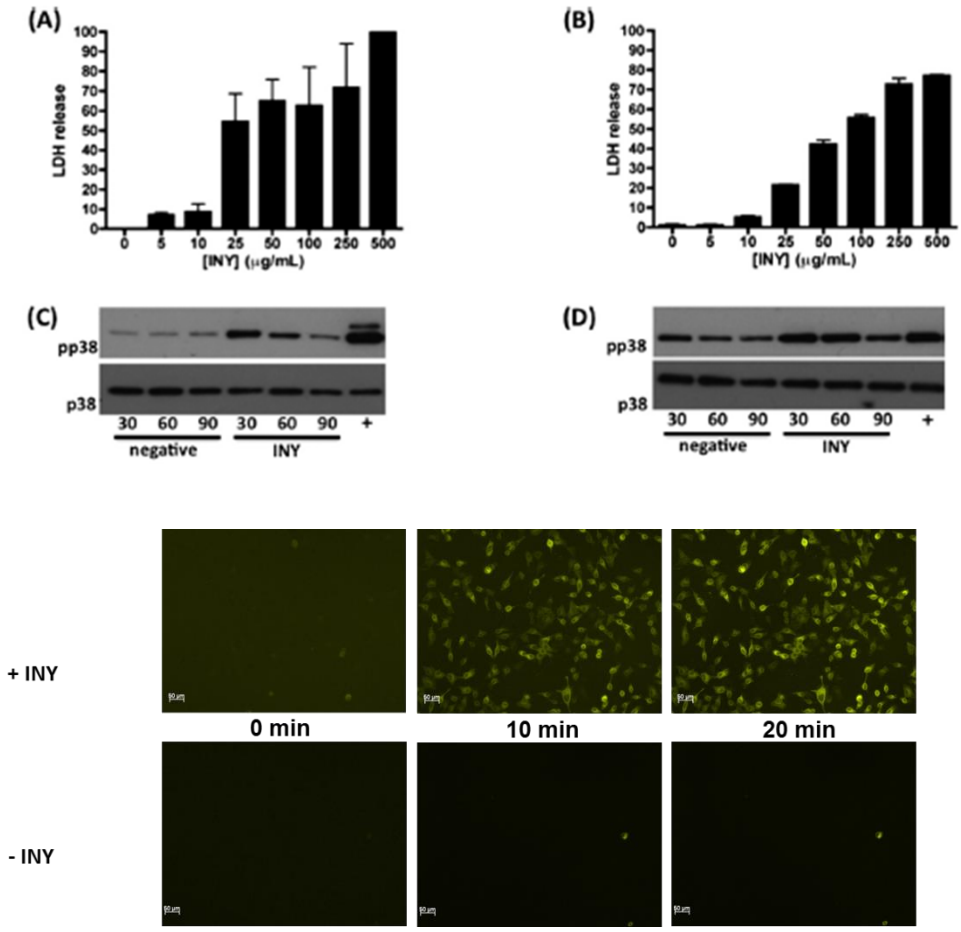


Figure 2.6 Recombinant INY can lyse and form functional pores leading to epithelial cell lysis and activates pro-inflammatory signaling. (A) HeLa cells were incubated with increasing concentrations of recombinant INY and epithelial cell lysis was measured by LDH release. Values were normalized to 100% lysis using Triton X-100 **(B)** HeLa cells were incubated with media alone (negative) or INY (3 μg/mL) for 30, 60 and 90 min. Cells were lysed and probed with phospho-p38 antibody (top panel) and total p38 antibody (bottom panel). HeLa cells were incubated with PLY as a positive control. **(C)** COS-7 cells were incubated with increasing concentrations of recombinant INY and epithelial cell lysis was measured by LDH release. **(D)** COS-7 cells were were incubated with media alone (negative), INY (3 μg/mL) for 30, 60 and 90 min. Cells were lysed and probed with phospho-p38 antibody (top panel) and total p38 antibody (bottom panel). COS-7 cells were incubated with PLY (0.8 ng/ml) as a positive control. **(E)** HeLa cells were incubated with fluorescently labelled phalloidin (final concentration of 3.3 nM) in the presence or absence of INY (final concentration of 1.8 μg/mL) for 30 minutes. Internalized phalloidin was detected by fluoresence microscopy. Scale bars represent 50 μm

2.4 Discussion

The vaginal mucosa is home to a complex ecosystem, with a microbiota that may remain stable over time or may undergo profound and rapid shifts, leading to disease states such as BV. Culture-based studies formed the foundation of our understanding of the vaginal microbiota, but more recent nucleic acid-based culture-independent investigations have deepened our understanding of vaginal ecology[60, 122, 123]. Most vaginal lactobacilli exert a protective effect and provide resistance to colonization by pathogens via the production of several anti-microbial substances. *L. iners* is an atypical organism recently identified as a member of the vaginal microbiota and may have pathogenic rather than (or perhaps in addition to) protective effects. We have characterized INY, a CDC family member and the first candidate virulence factor for *L. iners*. At the primary amino acid sequence level, INY is most similar to the human-specific CD59-dependent toxins VLY and ILY; however, its lytic activity is neither species nor cell type specific. A putative CD59 binding site has been reported for ILY [131], but this sequence is not present in VLY. Thus, an overall basis for CDC host specificity has not been defined. INY represents the nearest neighbor of the CD59-dependent CDCs and may serve as a useful tool for further evolutionary and functional comparisons. A hallmark of the unique biology of *L. iners* is its ability to continue to colonize the vagina under conditions under which other lactobacilli cannot, including during BV[36, 132]. INY is a thiol-activated, cholesterol-inhibitable toxin that is secreted by growing L. iners. Sublytic concentrations of INY activate p38 phosphorylation in human genital tract epithelial cells. This is a conserved and tightly regulated response to membrane disruption and osmotic stress[133, 134]. INY pores are sufficient to allow entry of

molecules from the extracellular space into the cytoplasm, as demonstrated with fluorescent phalloidin (Fig. 6). This effect may be of particular importance in immune responses to polymicrobial colonization[109, 135]. Cytolysin production may provide defensive functions by killing professional immune cells, may make available new anatomic niches by disrupting epithelial barriers, or may allow bacteria to access sequestered sources of nutrients such as iron from within erythrocytes. We hypothesize that CDC secretion, a feature common to *G. vaginalis* and *L. iners*, may be an important factor in survival in the inhospitable environment of BV, though at the highest extremes of vaginal pH (6.0), INY may be inactive. Human α-defensins, which inactivate CDCs[136], are decreased during BV[137], and this deficiency may allow INY-mediated cellular damage to occur. Antibodies against other CDCs, including PLY and VLY, may bind and inhibit INY, as may host antimicrobial peptides. Cauci et al. showed that local antitoxin IgA responses were correlated with mucosal cytokine levels and with BV diagnosis[138, 139]. Cross-reactivity between anti-VLY and anti-INY immunoglobulins may have implications for the efficiency of the host response to BV and for resolution of disease. The reversible deficiency of antimicrobial peptides observed in BV[137] may be important because of their antitoxin, as well as their antimicrobial, effects. Continued investigation of INY may help shed light on CDC evolution and on the role of *L. iners* in vaginal health and disease.

Chapter 3: Investigation into the pH dependent activity of INY

3.1 Introduction

The CDCs are a family of toxins produced by a wide range of organisms. These pore forming toxins can be crucial for pathogenesis of various organisms requiring pore formation. Functional pore formation results in membrane disruption, a consequence which can be manipulated for a number of various functions including destruction of immune cells, translocation of bacterial effectors into a eukaryotic cell[117], as well as phagosomal escape[104, 140]. Interestingly, these toxins which are produced by organisms with an intracellular component of their lifestyle display optimal/enhanced activity at acidic pHs consistent with their use in the acidic phagosome. The listerial derived CDCs (including LLO) are particularly well suited to their role as an escape mechanism from the phagosome, displaying significant activity at acidic pHs and rapidly losing activity at neutral pH. This elaborate and specific mechanism ensures that once the toxin mediates escape from the phagosome, it will maintain the integrity of the cell allowing intracellular replication of the bacteria. The molecular basis for this pH dependent activity has been localized to three residues in domain 4 of the protein, which act as a sensor for increased pH and trigger premature unfolding of the transmembrane regions and subsequent inactivation of the protein[103]. For a time, it was thought that pH dependent activity was specific only to *Listeria* derived toxins and that all other toxins were "pH insensitive"[141]. While such drastic pH dependence has only been shown for LLO, further studies demonstrated that PFO also displays a pH optimum, with greatly enhanced activity at pH 5.5-6 (although still able to function at neutral pH, unlike the *Listeria* derived

CDCs)[142]. Studies of PFO have suggested that at low pH, a partial unfolding event occurs, which primes the protein for membrane insertion.

The data on PFO and LLO suggest that CDCs may have evolved mechanisms which make them well suited to their site of action, the acidified phagosome. Given that *L. iners* can persist in a normal and BV states, where the pH can change from acidic to more neutral, we sought to understand how pH would affect the activity of its pore forming toxin, inerolysin[143]. Here, we demonstrate that INY shows a pH optimum, with significantly greater hemolytic activity at pH 4.5 than 7.4. We also attempt to understand the molecular basis for this pH dependent activity. Our work here suggests that pH dependent activity may be common to the family of CDCs, making certain toxins well suited to their respective physiological niches.

3.2 Materials and Methods

3.2.1 Cloning and expression of CDCs

The listeriolysin O (LLO) ORF lacking its predicted signal sequence was amplified by PCR from *L. monocytogenes* BAA751 genomic DNA using primers BamHI-LLO-F (GCCGCCGGATCCAAGGATGCATCTGCATTC AATAAAG) and LLO-R-XhoI (GCCGCCCTCGAGTTATTCGATTGGATT ATCTAC). The ILY ORF lacking its predicted signal sequence was amplified from *S. intermedius* ATCC 27335 genomic DNA using primers BamHI-ILY-F (GCCGCCGGATCCGCATTCGCTGAAA CACCTACC) and ILY-R-XhoI (GCCGCCCTCGAGTTAATCAGTGTTATC TTTCAC). Amplifications were performed using Phusion proofreading polymerase (New England BioLabs). A vector encoding a codon-optimized VLY

sequence (predicted amino acid sequence identical to that of the VLY from *G. vaginalis* ATCC 14018 lacking the signal sequence, codon optimized for expression in *E. coli*) flanked by NdeI and XhoI restriction sites was purchased from GenScript. For the creation of the INY double mutant, primers were used to replace the threonine at position 200 to an glutamate using primer pair primers NheI-INY-F (GCCGCCGCTAGCA ATACTGAGCCAAAAACAGCTATTG) and INY_Thr200_GluR(GCTTTGAGCACTTTCTGTATCATATTCAATTC) and INY_Thr200_GluR(GAATTGAATATGATACAGAAAGTGCTCAAAGC) and XhoI-INY-R (GCCGCCCTCG AGTTAGTCATTTTTTACTTCTTCTTTG). This was followed by replacement of a serine residue at position 239 with to a glutamate using NheI-INY-F (GCCGCCGCTAGCA ATACTGAGCCAAAAACAGCTATTG) and INY_Ser239_GluR (CTTAAAGTTAACAATTTCTGCTTGTTTCTC) and INY_Ser239_GluF (GAGAAACAAGCAGAAATTGTTAACTTTAAG) and XhoI-INY-R (GCCGCCCTCG AGTTAGTCATTTTTTACTTCTTCTTTG). The INY mutant showing reduced oligomerization capability (L508D) was created by introduction of the point mutation by PCR amplification from *L. iners* DSM13335 genomic DNA using primers NheI-INYL508D0F(GCCGCC**GCTAGC**AATACTGAGCCAAAAACAGCTATTG) and INYL508D-R-XhoI(GCCGCC**CTCGAG**TTAGTCATTTTTTACTTCTTCTTTGTGTTTAGGTCTATCAGTCGTACCC). Restriction sites are underlined and in bold. Introduction of the leucine to aspartate mutation is underlined. Protein purification was done as previously described in Chapter 2

3.2.2 Erythrocyte lysis assay

Preparation of erythrocytes was carried out as previously described in Chapter 2. To measure hemolysis, supernatant was removed, and the optical density at 415 nm (OD415) was

measured. Hemolysis assays were carried out in the presence of buffer C (35 mM sodium phosphate, 125 mM sodium chloride) as previously described[103]. Toxins were preincubated at the indicated pH for 30 min before use in an endpoint assay. In the indicated experiments, toxin was treated for 30 minutes at the indicated pH and then adjusted using HCl or NaOH and incubated for an additional 30 minutes at 4^{o}C before use in an endpoint assay.

3.2.3 Fluorescence spectroscopy

Unfolding of LLO and INY was measured over time by monitoring the change in 1-anilinonaphthalene-8-sulfonic acid (ANS; Molecular Probes) fluorescence [103]. Purified LLO or INY was diluted to a final concentration of 1M in 2 ml of buffer C (35 mM sodium phosphate, 125 mM sodium chloride) in a quartz cuvette. Fluorescence was monitored continuously for 30 min in a spectrophotometer with excitation and emission wavelengths set to 371 nm and 483 nm, respectively.

3.2.4 Binding activity of recombinant CDCs

Recombinant CDCs (5µg/mL) were incubated for 30 min at 37^{o}C at the either pH 4.5 or pH 7.4. 400µl toxin was mixed with 400µl of 1% hRBCs on ice for 5 min or with a 1% solution of VK2 cells for 20 min at 4^{o}C in Buffer C (35 mM sodium phosphate, 125 mM sodium chloride) at the indicated pH. Cells were recovered by centrifugation (9000rpm for 10 min at 4^{o}C). Cells were washed twice with ice cold PBS, and resuspended in 2X SDS buffer + 1% TX-100. Bound CDC was detected by western blot analysis using an anti-His-tag antibody (Sigma).

3.2.5 Binding of recombinant CDCs to immobilized cholesterol

To assess the binding of CDCs to cholesterol, a PVDF binding assay was carried out. Briefly, 20 mg/mL cholesterol (dissolved in 1:1 chloroform:ethanol) was diluted to 20 µg/mL (dissolved in 1:5 chloroform:ethanol), and 100 µl was added to each well (2 µg per well) of a 96 well plate with Immobiolon-P membrane (Millipore) at the bottom and allowed to dry overnight. The cholesterol-coated wells were treated with blocking buffer consisting of 4% heat-inactivated fetal bovine serum in PBS for 1h. CDCs at 2 µg/mL were treated for 30 min at either 37^{o}C or 4^{o}C at in Buffer C (35 mM sodium phosphate, 125 mM sodium chloride) at pH 4.5 or pH 7.4. Toxin was diluted to 2nM in Buffer C with 4% heat inactivated fetal bovine serum and 100 µl was added to each well. After 2 hours, wells were washed four times with blocking buffer, and treated with anti His-tag antibody (1:2000) for 1 hour. Wells were washed four times with blocking buffer and antibody was detected with horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz) at a dilution of 1:2000. The binding of CDCs to cholesterol was determined quantitatively by the addition of 100 µl of 3,3β,5,5β-tetramethylbenzidine (TMB). The reaction was stopped by the addition of 50 µl of 2N H_2SO_4 and absorbance was measured at 450nm. For INY and LLO, percent binding is measured relative to toxin treated at pH 4.5 and 4^{o}C. For PLY, percent binding is measure relative to toxin treated at pH 7.4 and 4^{o}C.

3.2.6 Protein degradation Assay

Protein degradation was assessed by western blot analysis. Briefly, CDCs (5 µg/mL) were treated for 37^{o}C in buffer C (35 mM sodium phosphate, 125 mM sodium chloride) at pH 4.5 or 7.4 for 30 min. Protein levels were detected by western blot analysis using anti-his-tag

antibody. Primary antibody was detected using horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz) at a dilution of 1:1000 and detected by enhanced chemiluminesence.

3.2.7 SDS agarose gel electrophoresis separation of CDC monomer and oligomer

SDS-AGE was carried out as described[144]. Briefly, 60 µg/mL of toxin was mixed with 1% hRBCs, in a final volume of 40 µl and incubated at 37^{o}C for 30 min. After 30min, glutaraldehyde was added to a final concentration of 5mM and incubated for an additional 2 min at room temperature. Samples were solubilized SDS sample buffer and the complexes were analyzed on a 1.5% SDS-agarose gel (100 V, 120 min) and then transferred to nitrocellulose membranes. Protein bands were identified using mouse anti-his-tag antibody (Sigma) followed by horseradish peroxidase tagged goat anti-mouse secondary IgG. The bands were visualized using enhanced chemiluminesence (ECL Western Blotting Detection Reagents, Roche).

Table 3.1 Primers used for INY domain swap

Domain Fusion	Name	Sequence
PLYD1-3/INYD4	INY_D4_XHOI_R	GCCGCCCTCGAGTTAGTCATTTTTTACTTCTTCTTTGTG
	INY_D4_F	GTTGAGACTAAGGTTACAGCTTACAGAAATGGTAAATTGATATTGAACCATAAAG
	PLYD1-3_NdeI F	GCCGCCCATATGATGGCAAATAAAGCAGTAAATGAC
	PLYD1-3_R	CTTTATGGTTCAATATCAATTTACCATTTCTGTAAGCTGTAACCTTAGTCTCAAC
PLYD1-2/INYD3/PLYD4	PLYD1-2_NDEI_F	GCCGCCCATATGATGGCAAATAAAGCAGTAAATGAC
	PLY_1-147_R	TTGAGCACTAGTTGTATCATATTCAATTCTAGCTGGGACATTATTGACC
	INY_196-245_F	GGTCAATAATGTCCCAGCTAGAATTGAATATGATACAACTAGTGCTCAA
	INY_196-245_R	GCGTCTACGCTGACTGTATAATAAATTTGCTTAAAGTTAACAATTGATGC
	INY_292-366_F	TATTTCGAGTGTTGCTTATGGGCGTTCTATGTACATTAAGTTGGAAAC
	INY_292-366_R	GGATGATCTGCTGTAAAGCGGCTACCTTTTTGAATTAATTCTTTCA
	PLY_319-end_F	TGAAAGAATTAATTCAAAAAGGTAGCCGCTTTACAGCAGATCATCC
	PLYD4_XHOI_R	GCCGCCGCTAGCCTAGTCATTTTCTACCTTATCCTCTACC
PLYD1/INYD2/PLYD3-4	PLYD1-2_NDEI_F	GCCGCCCATATGATGGCAAATAAAGCAGTAAATGAC
	PLY_1-22R	GTTAACATGTCACCTTGTCTTGTCAAGAGTTTCTTTTTATCGTAATTCAT
	INY_69-105F	ATGAATTACGATAAAAAGAAACTCTTGACAAGACAAGGTGACATGTTAAC
	INY_69-105R	GTAGCTGTTACAGAAATATCACTTGTACTGTTAGAAATGGTCTTCTTTTGT
	PLY_57-343F	ACAAAAGAAGACCATTTCTAACAGTACAAGTGATATTTCTGTAACAGCTAC
	PLY_57-343R	AATCAGCGGTATTGTTGATTACCGCAACTACATTGTCACGTA
	INY_391-407F	TACGTGACAATGTAGTTGCGGTAATCAACAATACCGCTGATT
	INY_391-407R	ATCCAGCAGTAAATCTCCGTTTGAATATTCAGTTACTTTAGTAGCAAT
	PLY_359-endF	ATTGCTACTAAAGTAACTGAATATTCAAACGGAGATTTACTGCTGGAT
	PLYD4-XHOI	GCCGCCGCTAGCCTAGTCATTTTCTACCTTATCCTCTACC
INYD1/PLYD2-4	INY__1-68F NHEI	GCCGCCGCTAGCAATACTGAGCCAAAAACAGCTATTG
	INY_1-68R	AATACTTTCTCCCTGATGGGTCAAAACAGCTTGTGAATCATAAT
	PLY_22-57F	ATTATGATTCACAAGCTGTTTTGACCCATCAGGGAGAAAGTATT
	PLY_22-57R	CATTAGCAGTAGTTACTGAAATACTTGATGTATTTGTCGACAAGCTCC
	INY_106-195F	GGAGCTTGTCGACAAATACATCAAGTATTTCAGTAACTACTGCTAATG
	INY_106-195R	CCGTTATTTTTTCATACTGCATTCTGGCAGGAATGGCGTGA
	PLY_148-197F	TCACGCCATTCCTGCCAGAATGCAGTATGAAAAAATAACGG
	PLY_148-197R	CATCAACACTGGCAGTGTAGTAAATCTGCTTAAAATTAACAATCTGAATC
	INY_246-291F	GATTCAGATTGTTAATTTTAAGCAGATTTACTACACTGCCAGTGTTGATG
	INY_246-291R	CAACTTGAGATAGACTTGGCGTCCATAAGATACACTAGAAACATAAACT
	PLY_244-318F	AGTTTATGTTTCTAGTGTATCTTATGGACGCCAAGTCTATCTCAAGTTG
	PLY_244-318R	ACCTGGGTTAGAACTATCAAATTTACTGCCTTCTTGAATCAAGTC
	INY_367-390F	GACTTGATTCAAGAAGGCAGTAAATTTGATAGTTCTAACCCAGGT
	INY_367-390R	CATAGTCTGTACTGTTTTGAAAGGTTAAAGGACAACCAAGTTGCA
	PLY_343-endF	TGCAACTTGGTTGTCCTTTAACCTTTCAAAACAGTACAGACTATG
	PLYD4-XHOI	GCCGCCGCTAGCCTAGTCATTTTCTACCTTATCCTCTACC

Table 3.2 Primers used for PLY domain swaps

Domain Fusion	Name	Sequence
INY D1-3/PLY D4	PLYD-4F_for INYD1-3 F	GATTATATTGCTACTAAAGTAACTGAATATTCAAACGGAGATTTACTGCTGGATC
	PLYD-4_XHOI R	GCCGCCCTCGAGCTAGTCATTTTCTACCTTATCCTCTACC
	INYD1-3F-NheI	GCCGCCGCTAGCAATACTGAGCCAAAAACAGCTATTG
	INYD1-3R	GATCCAGCAGTAAATCTCCGTTTGAATATTCAGTTACTTTAGTAGCAATATAATC
INYD1-2/PLYD3/INYD4	INY1-195F_NHEI	GCCGCCGCTAGCAATACTGAGCCAAAAACAGCTATTG
	INY 1-195R	GCCGTTATTTTTTCATACTGCATTCTGGCAGGAATGGCGTGA
	PLY 148-197F	TCACGCCATTCCTGCCAGAATGCAGTATGAAAAAATAACGGC
	PLY 148-197R	CATCAACACTGGCAGTGTAGTAAATCTGCTTAAAATTAACAATCTGAATCTG
	INY 246-291F	CAGATTCAGATTGTTAATTTTAAGCAGATTTACTACACTGCCAGTGTTGATG
	INY 246-291R	CAACTTGAGATAGACTTGGCGTCCATAAGATACACTAGAAACATAAACTAAAG
	PLY 244-318F	CTTTAGTTTATGTTTCTAGTGTATCTTATGGACGCCAAGTCTATCTCAAGTTG
	PLY244-318R	CACCTGGGTTAGAACTATCAAATTT
	INY 367-end F	GGACTTGATTCAAGAAGGCAGTAAATTTGATAGTTCTAACCCAGGTG
	INY_D4_XHOI_R	GCCGCCCTCGAGTTAGTCATTTTTTACTTCTTCTTTGTG
INYD1/PLYD2/INYD3-4	INY1-195F_NHEI	GCCGCCGCTAGCAATACTGAGCCAAAAACAGCTATTG
	INY1-68R	CAATACTTTCTCCCTGATGGGTCAAAACAGCTTGTGAATCATAATC
	PLY22-57F	GATTATGATTCACAAGCTGTTTTGACCCATCAGGGAGAAAGTATTG
	PLY22-57R	TCATTAGCAGTAGTTACTGAAATACTTGATGTATTTGTCGACAAGCTCC
	INY106-390F	GGAGCTTGTCGACAAATACATCAAGTATTTCAGTAACTACTGCTAATGA
	INY 106-390R	CATAGTCTGTACTGTTTTGAAAGGTTGCAACTTGGTTGTCCTTTAAG
	PLY343-359F	CTTAAAGGACAACCAAGTTGCAACCTTTCAAAACAGTACAGACTATG
	PLY343-359R	CTTTATGGTTCAATATCAATTTACCATTTCTGTAAGCTGTAACCTTAGTCT
	INY 408-endF	AGACTAAGGTTACAGCTTACAGAAATGGTAAATTGATATTGAACCATAAAG
	INY_D4_XHOI_R	GCCGCCCTCGAGTTAGTCATTTTTTACTTCTTCTTTGTG
PLYD1/INYD2-4	PLY1-21FNHEI	GCCGCCGCTAGCATGGCAAATAAAGCAGTAAATGAC
	PLY1-21R	TTGTTAACATGTCACCTTGTCTTGTCAAGAGTTTCTTTTTATCGTAATTCATAGC
	INY69-105F	GCTATGAATTACGATAAAAAGAAACTCTTGACAAGACAAGGTGACATGTTAACAA
	INY69-105R	TTGGTAGCTGTTACAGAAATATCACTTGTACTGTTAGAAATGGTCTTCTTTTG
	PLY58-147F	CAAAAGAAGACCATTTCTAACAGTACAAGTATATTTCTGTAACAGCTACCAA
	PLY58-147R	CTTTGAGCACTAGTTGTATCATATTCAATTCTAGCTGGGACATTATTGACC
	INY196-245F	GGTCAATAATGTCCCAGCTAGAATTGAATATGATACAACTAGTGCTCAAAG
	INY196-245R	GCGTCTACGCTGACTGTATAATAAATTTGCTTAAAGTTAACAATTGATGCT
	PLY198-243F	AGCATCAATTGTTAACTTTAAGCAAATTTATTATACAGTCAGCGTAGACGC
	PLY198-243R	TTGTTTCCAACTTAATGTACATAGAACGCCCATAAGCAACACTCGAAATATA
	INY292-366F	TATATTTCGAGTGTTGCTTATGGGCGTTCTATGTACATTAAGTTGGAAACAA
	INY292-366R	GGATGATCTGCTGTAAAGCGGCTACCTTTTTGAATTAATTCTTTCAA
	PLY319-342F	TTGAAAGAATTAATTCAAAAAGGTAGCCGCTTTACAGCAGATCATCC
	PLY319-342R	AATATAATCAGCGGTATTGTTGATTACCGCAACTACATTGTCACGTAAA
	INY391-endF	TTTACGTGACAATGTAGTTGCGGTAATCAACAATACCGCTGATTATATT
	INY_D4_XHOI_R	GCCGCCCTCGAGTTAGTCATTTTTTACTTCTTCTTTGTG

3.2.8 Transmission Electron Microscopy

Erythrocyte ghost membranes were made by placing 10 µl of a 50% hRBC cell suspension in a drop of water and incubated at room temperature for 10 minutes to allow the membranes to rise to the air-liquid interface. Formvar coated 300 mesh grids (Electron Microscopy Sciences) were placed onto the drop and incubated for 5 min. INY (125 µg/mL) was pretreated in Buffer

C at pH 4.5 or pH 7.4 for 30 min and then a drop was placed onto the grid and incubated at 37^{o}C for 30 min. Grids were stained with 2% phosphotungstic acid for 30 sec. Samples were examined using a JEOL JEM-1200 EXII electron microscope. Pictures were taken on an ORCA-HR digital camera (Hamamatsu) and recorded with the AMT Image Capture Engine.

3.2.9 Construction of INY derivatives and chemical modification of cysteine residues with IANBD

Proteins were purified as indicated previously. Toxins were treated with 2.5mM DTT for 10 minutes at room temperature and then dialyzed overnight to remove DTT. Toxin derivatives were labeled with a 20-fold molar excess of the IANBD[iodoacetamido-N,N'-dimethyl-N-(7 nitrobenz-2-oxa-1,3-diazolyl)ethylene-diamine; Molecular Probes] overnight at 4^{o}C. Following modification with the probe, excess was quenched with 1mM DTT for 10 minutes and probe was removed via extensive dialysis. NBD measurements were carried out with an Infinite 200 microplate reader (Tecan) using the following settings: an excitation wavelength of 480nm and an emission wavelength of 540 nm with a bandpass of 5nm. Emission intensity was scanned between 500 and 600 nm at a resolution of 1 nm with an integration time of 1 sec. 10 µg of toxin was incubated with varying amounts of cholesterol in Buffer C for 30 minutes at 37^{o}C. The fluorescence intensity of the unlabeled samples was subtracted from that of the fluorescent probe-labeled samples in order to control for the intrinsic fluorescence of the sample in the absence of probe.

Table 3.3 Primers used for construction of cysteine mutants

Toxin	Primers	Sequence
INYC476A	INY-CysAla-F	GA ATG TTA AGA TTC AAG AAG CTA CAG GCT TGG CAT G
	INY-CysAla-R	CAT GCC AAG CCT GTA GCT TCT TGA ATC TTA ACA TTC A
INYD305C	INY-AspCys-F	GGA AAC AAC AAG CAA GAG TTG CAA AGT TCA AGC AGC TTT TG
	INY-AspCys-R	CAA AAG CTG CTT GAA CTT TGC AAC TCT TGC TTG TTG TTT CC
INYI339C	INY-IleCys-F	ACT AGT GTT GTA GCT GTT TGC TTA GGT GGT AAC TC
	INY-IleCys-R	GAG TTA CCA CCT AAG CAA ACA GCT ACA ACA CTA GT

3.2.10 Statistical methods

Hemolysis assay results are expressed as average values from three readings from one representative experiment. Each experiment was repeated three times. Data were analyzed by Student *t* test. A *P* value of <0.05 was considered significant.

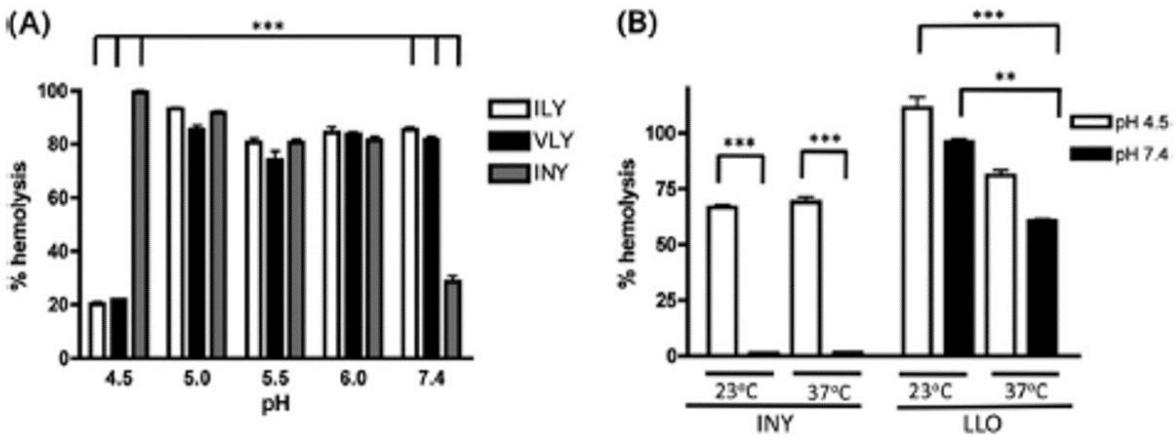
3.3 Results

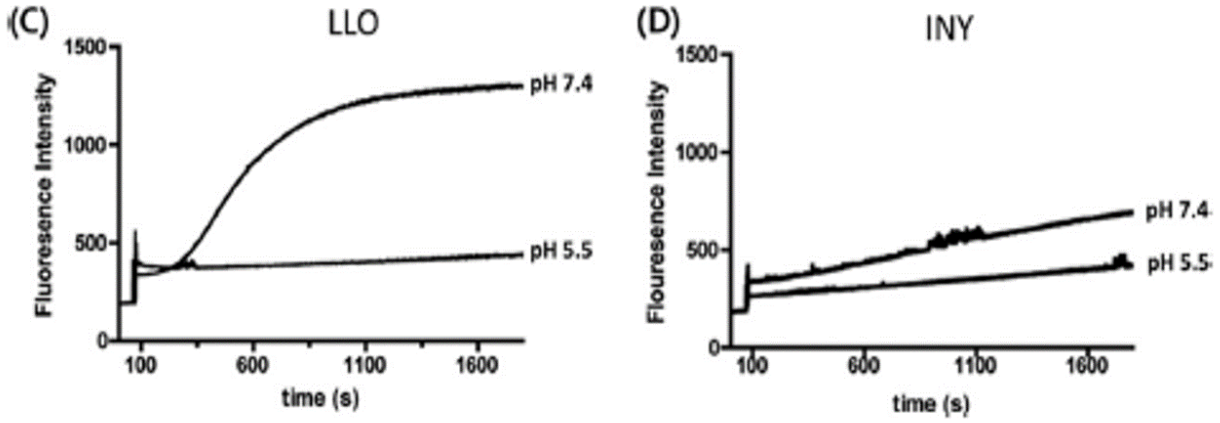
3.3.1 Recombinant INY shows pH dependent activity which is distinct from LLO

The CDCs have various pH-dependent activity profiles. ILY has activity across a wide pH range[128], while LLO (produced by *L*. *monocytogenes*) has an acidic pH optimum, consistent with its function within a vacuole during the *Listeria* life cycle[103]. The hemolytic activity of INY was measured as a function of pH and compared to the hemolytic activities of several other CDCs (Fig. 1A). ILYand VLY were both active at neutral pH and demonstrated a significant

decrease in activity at pH 4.5. This reduction in ILY activity at acidic pH was consistent with a previous report[128]. In contrast, INY demonstrated greater activity at a more acidic pH (4.5 to 6.0), with decreased function at pH 7.4 (Fig 3.1A). The molecular basis of pH dependence has been extensively investigated for LLO[103]. To gain further insights into the pH-dependent activity of INY, we compared the activity of INY to that of LLO as a function of both pH and temperature. It has been previously shown that LLO responds to a pH increase by rapid unfolding and loss of activity. Maximal unfolding of LLO requires increases in both temperature and pH. With an increased pH, INY rapidly lost activity at both 23°C and 37°C (Fig 3.1B). This is in contrast to LLO, which showed a slight decrease with an increase in either pH or temperature and a more pronounced decrease in activity upon incubation at increased pH and increased temperature. Toxin unfolding in response to pH, as has been demonstrated for LLO, was measured using the fluorescent probe ANS, which binds hydrophobic sites on proteins. Addition of LLO to a solution of ANS led to an increase in fluorescence (Fig 3.1C) that was considerably more pronounced at pH 7.4. In contrast, INY demonstrated only a slight increase in fluorescence over time at pH 7.4, as well as pH 5.5 (Fig 3.1D). To further distinguish the mechanism of pH dependent activity of INY, toxins were incubated at either pH 4.5 or pH 7.4 for 30 minutes. The pH was then shifted back to pH 4.5 followed by an additional 30 minute incubation. Once LLO was inactivated by elevated pH, shifting the pH could not restore hemolytic activity. Interestingly, the loss of activity seen with INY was reversible, and upon shifting the pH we see a restoration of hemolytic activity to levels comparable to that of pH 4.5 treated toxin. For LLO, a triad of acidic residues in domain 4 is responsible for sensing increased pH and driving unfolding of the protein. In order to determine if the analogous residues in INY

were responsible for this unique pH dependent activity, they were mutated in order to resemble LLO. In LLO three residues comprise this “acidic sensor”: Glu-208, Glu-247, and Asp 320. Fig 3.1F is a protein sequence alignment, highlighting the analogous residues in INY: Thr-201, Ser-239, and Asp-312. Inserting the triad of acidic sensor residues into INY was not sufficient to confer LLO-like activity on the protein and this recombinant mutant toxin maintained INY-like activity (Fig 3.1G).




(C)
LLO
Fluorescence Intensity
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1000
500
0
100
600
1100
1600
time (s)
pH 7.4
pH 5.5
(D)
INY
Flouresence Intensity
1500
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100
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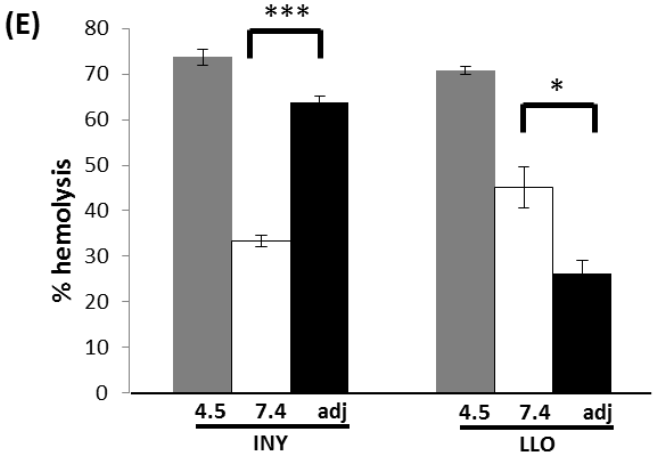
1100
1600
time (s)
pH 7.4
pH 5.5


(E)
% hemolysis
80
70
60
50
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*
4.5
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(F)
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SAKIDYD EI AYSESQLIAK
PARIEYD TS AQSMDQLKVK
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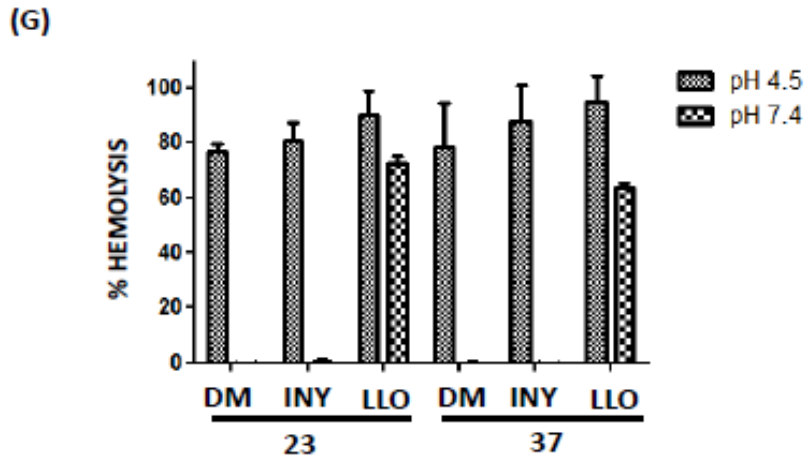


Figure 3.1 **Recombinant INY shows pH dependent activity distinct from that of LLO (A)** INY, ILY, VLY and LLO (125ng/mL) were incubated with human erythrocytes under varying pH conditions at 37°C. Percent hemolysis was calculated from OD_{415} of supernatant after 45 min. These values were normalized to 100% lysis using Triton X-100 **(B)** INY and LLO (125ng) were preincubated at the indicated temperature for 20 minutes before used in an erythrocyte lysis assay at the indicated temperature. **(C)** The hydrophobicity of INY and **(D)** LLO was determined over time by measuring the binding of fluorescent probe ANS. 1µM of each toxin was injected into buffer containing 50µM ANS 100s after starting data acquisition. ANS fluoresence intensity was measured over time at 481nm **(E)** INY and LLO were treated at the indicated pH for 30 min at 37°C. For the adjusted (adj) sample, the pH of toxin treated at pH 7.4 for 30 min was adjusted to pH 4.5 and incubated for an additional 30 minutes at 4°C and then used in a hemolysis assay. **(F)** Protein alignment of regions surrounding the residues involved in the pH dependent activity **(G)** Residues in INY were mutated to look like LLO and used in a hemolysis assay. *P values* for this and subsequent figures are as follows * <0.05 **<0.01 ***<.001

3.3.2 Membrane Binding activity of pH treated CDCs

We examined the membrane binding activity of toxins at the indicated pH to determine if this first step was impaired at more basic pH. There was no loss of membrane binding activity to

either hRBCs (Fig 3.2A) or VK2 epithelial cells (Fig 3.2B) under basic conditions for INY. This is in contrast to LLO, which shows a significant loss of membrane binding activity. The cholesterol dependent cytolysin (CDC) family is characterized by a dependence on cholesterol for hemolytic activity. For most (with the exception of the human specific CDCs ILY and VLY), membrane cholesterol serves as the receptor to which they initially bind[145]. We determined whether cholesterol binding activity was intact after incubation under basic conditions. INY and LLO were treated at pH 4.5 or pH 7.4 for 30 min at 37^{o}C. The amount of CDCs bound was then measured by ELISA using an anti-his-tag antibody. After treatment of INY at pH 7.4, its binding was comparable to binding of toxin treated at pH 4.5 (Fig 3.2C). Again, this is contrast to LLO, where treatment at pH 7.4 resulted in a significant reduction in cholesterol binding ability as compared to binding at pH 4.5. Protein levels were assessed by western blot to ensure that the unique pH dependent activity was not due to protein degradation. We observed comparable protein levels between toxin treated at pH 4.5 and toxin treated at pH 7.4 (Fig 3.2D).

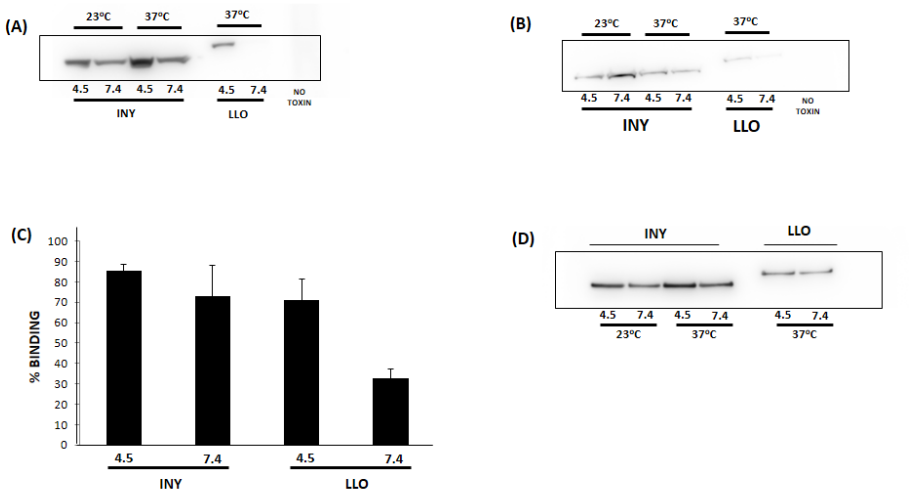


Figure 3.2 Membrane binding activity is intact and loss of activity is not due to protein degradation (A) INY and LLO (100ng/mL) were pretreated at 37°C for 30 minutes at the indicated pH. These toxins were then incubated with human RBCs for 5 minutes on ice and then membrane binding was assessed by western blot. **(B)** INY and LLO (100ng/mL) were pretreated at 37°C for 30 minutes at the indicated pH. These toxins were then incubated with HeLa cells for 20 minutes at 4°C and then membrane binding was assessed by western blot **(C)** Cholesterol 2.00 mg per well) was immobilized on a PVDF membrane well. His-tagged CDCs treated at 4.5 or 7.4 at 4°C or 37°C (black bars) were placed in the cholesterol-immobilized PVDF well and incubated for 1 h. After incubation, wells were washed and the binding of CDCs to cholesterol was analyzed by ELISA using anti his-tag antibody. **(D)** INY and LLO were were pretreated at 37°C for 30 minutes at the indicated pH. Intact protein levels were assessed by western blot

3.3.3 Several CDCs show pH dependent activity

To date, pH dependent activity has only been described for the *Listeria* derived toxins[141] as well as PFO[142]. Given that PLY is the most closely related non-species specific CDC, we examined the pH dependent activity of this toxin. The hemolytic activity of PLY at pH 4.5 and pH 7.4 was measured across a range of concentrations and compared to INY. The pH dependent profile of PLY is reversed as compared to INY, showing a loss of hemolytic activity at

pH 4.5 as compared to pH 7.4 (Fig 3.3A). With the finding that both INY and PLY demonstrate pH dependent activity, we expanded the panel of CDCs tested to include arcanolysin (ALN) and pyolysin (PLO), produced by *Arcanobacterium haemolyticum[146]* and *Arcanobacterium pyogenes[126]* respectively. Phylogenetically divergent from INY, these toxins also demonstrated pH dependent activity. ALN showed enhanced activity at pH 7.4 while PLO had enhanced activity at pH 4.5 (Fig 3.3 B). PLY was either preincubated for 30min at 37^{o}C at pH 4.5 or pH 7.4. The pH of the sample was then adjusted to pH 7.4 and incubated for an additional 30 min at 4^{o}C. Similar to INY, PLY showed a loss of hemolytic activity (at pH 4.5), which was restored after the pH shift. Like INY, this loss of hemolytic activity was not due to protein degradation (Fig 3.3E) at pH 4.5. Binding of PLY to erythrocytes (Fig 3.3C) and cholesterol (Fig 3.3D) was comparable between both pH treatments.

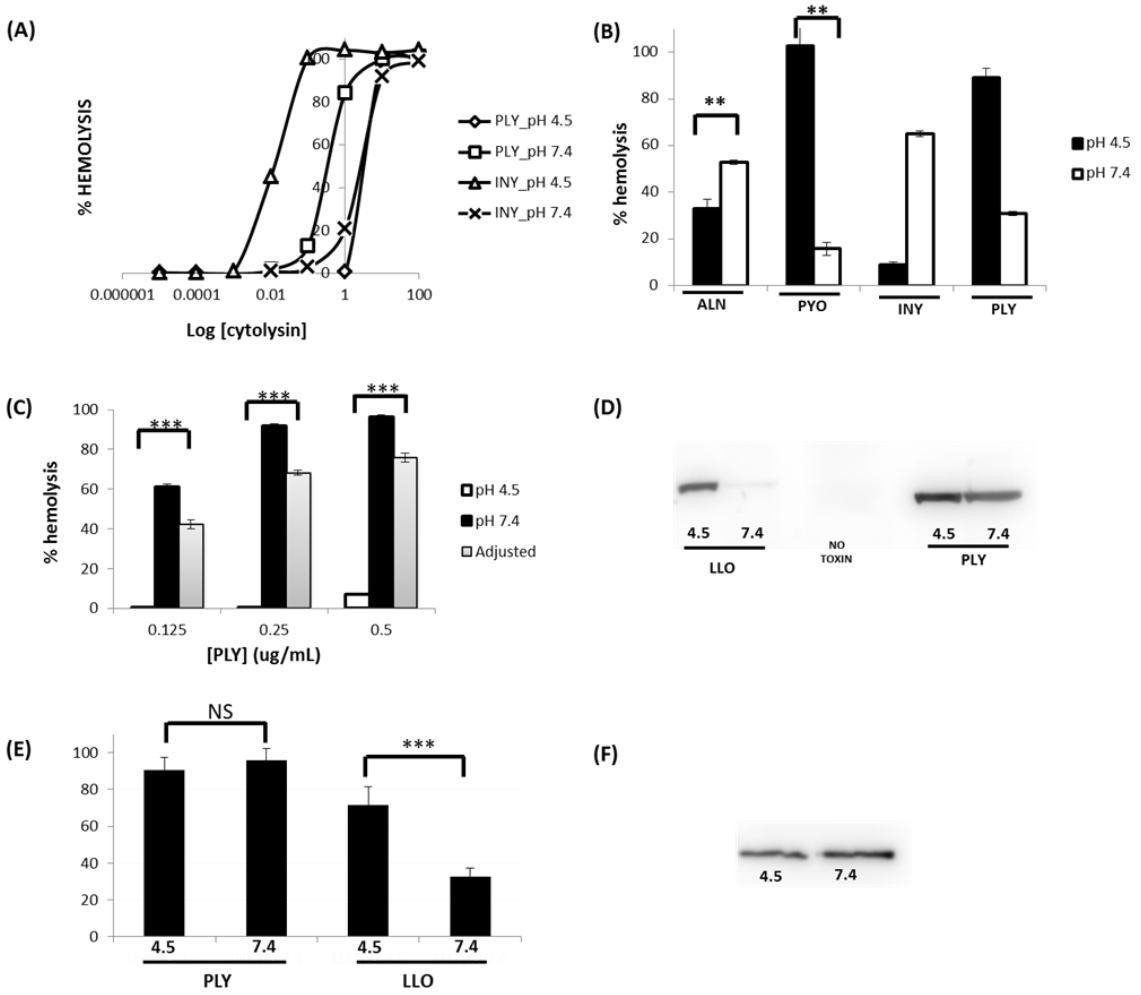
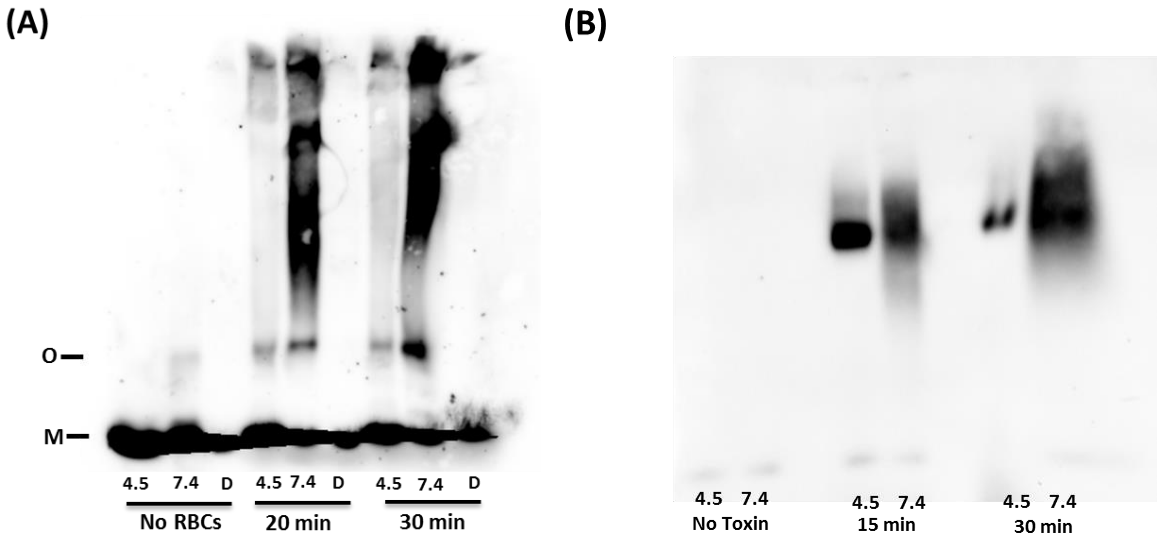


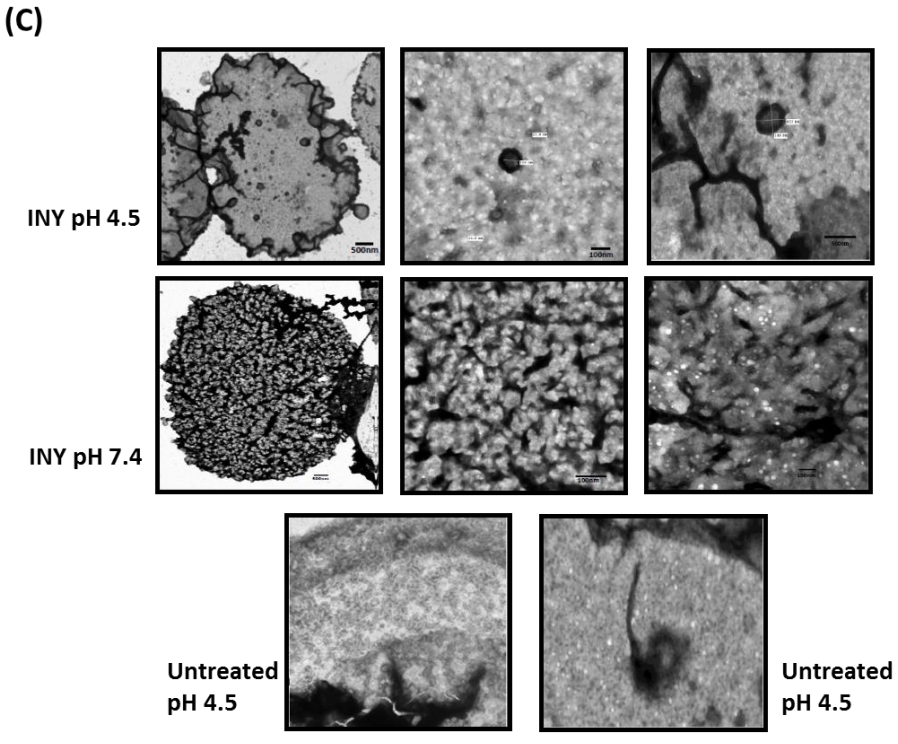
Figure 3.3 Several CDCs show pH dependent activity (A) PLY and INY were incubated at 37°C for 30 min and then used in an endpoint hemolysis assay **(B**) ALN, PLO, INY, and PLY (125ng/mL) were incubated at 37oC for 30 min at the indicated pH and used in an endpoint hemolysis assay (**C)** PLY was treated at the indicated pH for 30 min at 37°C. For the adjusted sample, the pH of toxin treated at pH 4.5 for 30 min was adjusted to pH 7.4 and incubated for an additional 30 minutes at 4°C and then used in a hemolysis assay. **(D)** PLY and LLO were pretreated at 37°C for 30 minutes at the indicated pH. These toxins were then incubated with human RBCs for 5 minutes on ice and then membrane binding was assessed by western blot **(E)** Cholesterol (2.00 mg per well) was immobilized on a PVDF membrane well. His-tagged CDCs treated at 4.5 or 7.4 at 4°C or 37°C (black bars) were placed in the cholesterol-immobilized PVDF well and incubated for 1 h. After incubation, wells were washed and the binding of CDCs to cholesterol was analyzed by ELISA using anti his-tag antibody **(F)** PLY was pretreated at 37°C for 30 minutes at the indicated pH. Intact protein levels were assessed by western blot.

3.3.4 Oligomerization properties of pH treated toxin

After membrane binding, several structural changes occur which results in oligomerization of monomers into a prepore structure. These large oligomeric complexes, ready for pore formation, are notably SDS resistant[147]. Briefly, toxin (preincubated at the indicated pH at 37^{o}C for 30 min) was incubated with red blood cells (2 x 10^{8} cells/mL) for 30 min. Samples were then solubilized with SDS without heating and separated by SDS-agarose gel electrophoresis, which should separate monomeric and oligomeric forms. After 20 minutes, SDS resistant oligomers were detected at both pH treatments. However, at pH 7.4 we detected a greater number of oligomers as compared to toxin treated at pH 4.5 (Fig 3.4A). At pH 7.4, despite a loss of hemolytic activity, oligomerization can still occur. Interestingly, we also detected a higher molecular weight species present in the pH 7.4 treated toxin, which was not present in the pH 4.5 treated toxin. PLY demonstrated competence for oligomer formation at both pH treatments as well, similar to INY (Fig 3.4B). To further assess competence for oligomerization, we monitored this process on the surface of erythrocyte ghosts by transmission electron microscopy (TEM). For pH 4.5 treated toxin, we observed ring like structures on the surface of the erythrocyte ghosts. Notably, we observed some structures which possessed dimensions consistent with what has been see for other CDCs, but the majority of these pore structures were approximately 300-400nm in diameter, which is about 10 times greater than the size of pores observed for other known CDCs[148]. The presence of two species of pores has been observed for PFO previously, and it is yet unclear if these species are competent for pore formation[92]. At pH 7.4, no observable pores could be detected on the surface of the erythrocyte ghosts (Fig. 3.4C), despite the observation of high molecular weight structures in

our SDS-PAGE analysis. Additionally, we observed disorganized regions on the surface of the erythrocyte ghosts (Fig 3.4D) which is consistent with disorganized linearly bound toxin seen with oligomerization defective mutants of streptolysinO[149]. This pattern of dark staining is also consistent with monomeric bound toxin, as seen previously with PFO in solution[92]. As an additional assesment of competence for oligomerization, we also attempted to monitor structural transitions that mediate this process. Upon membrane binding, the rotation of β5 loop away from the β4 sheet contributes to the formation of an SDS resistant prepore, and this can be monitored spectroscopically using the environmentally sensitive probe conjugated to a cysteine residue substituted for ILE-339 in β4. Initially buried under the β5 sheet and in a nonpolar environment, this residue becomes exposed to the polar environment and fluorescence is quenched. At either pH, we observed a comparable reduction in fluorescence intensity, indicating the presence of these structural changes in both pH treated toxins. Taken together, our results indicate that at either pH, despite a loss of hemolytic activity, monomeric toxin was competent for oligomerization.


(A)
O
M
4.5 7.4 D
4.5 7.4 D
4.5 7.4 D
No RBCs
20 min
30 min
(B)
4.5 7.4
No Toxin
4.5 7.4
15 min
4.5 7.4
30 min


(C)
INY pH 4.5
INY pH 7.4
Untreated
pH 4.5
Untreated
pH 4.5

(C)

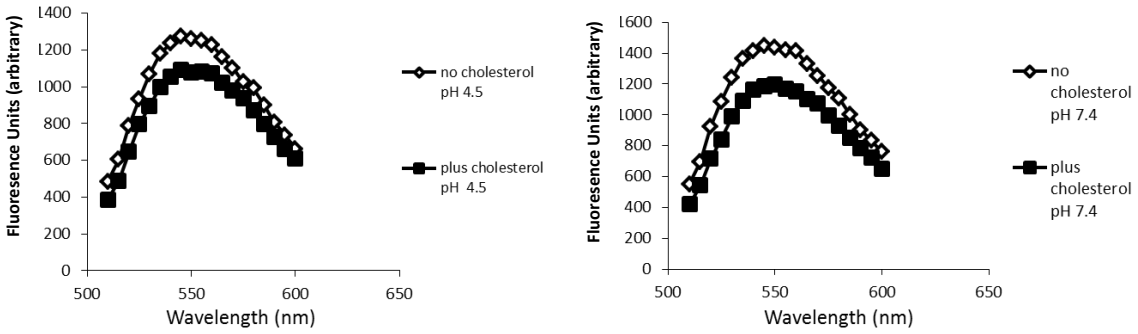


Figure 3.4 CDCs shows no defect in oligomerization after pH treatment. (A) Oligomerization of INY and L508D oligomerization defective mutant (D) on human RBCs (a final volume of 40ul) was determined using SDS-AGE and detected using anti His antibody. **(B)** Oligomerization of PLY on human RBCs (a final volume of 40ul) was determined using SDS-AGE and detected using anti His antibody. **(C)** TEM images of INY treated at the indicated pH **(D)** The mutant I339C was labeled with NBD and the fluoresence emission was measured in the absence of cholesterol and after incubation with cholesterol.

3.3.5 CDCs show a defect in membrane insertion after pH treatment

Having determined that at both acidic and neutral pH, membrane binding activity was intact and toxins are competent for oligomerization, we assessed the final step in pore formation, the conversion of a prepore to a pore and insertion of the transmembrane helices. In order to monitor these structural changes, we conjugated the environmentally sensitive probe NBD to a cysteine residue substituted for Asp-305 in TMH2. Upon incubation with cholesterol crystals, we found that at pH 4.5 there was a significant increase in fluorescence intensity (Fig. 3.5). In contrast, at pH 7.4, toxin incubated with cholesterol crystals showed no increase in fluorescence intensity, indicating a lack of insertion of transmembrane helices at this elevated pH (Fig. 3.5). We attempted to monitor several other steps in pore formation by substituting other residues with cysteine: His 232 (which monitors insertion of TMH1) and Gly 214 (which monitors disruption of the domain 2-domain 3 interface and unfurling of the transmembrane helices). For the Gly-214 mutant, toxin maintained only 20% of its WT activity making it

difficult to observe changes in fluorescence intensity. For His232, our labeling procedure seemed to be ineffective.

Table 3.4 INY and its derivatives

Toxin	Description	%WT activity
INYC476A	Recombinant native INY with an alanine substitution for the native cysteine (Cys-476) in the undecapeptide	50%
INYD305C	Recombinant INY with a cysteine substiution at position 305. This labels TMH2 and should undergo a polar to nonpolar transition	50%

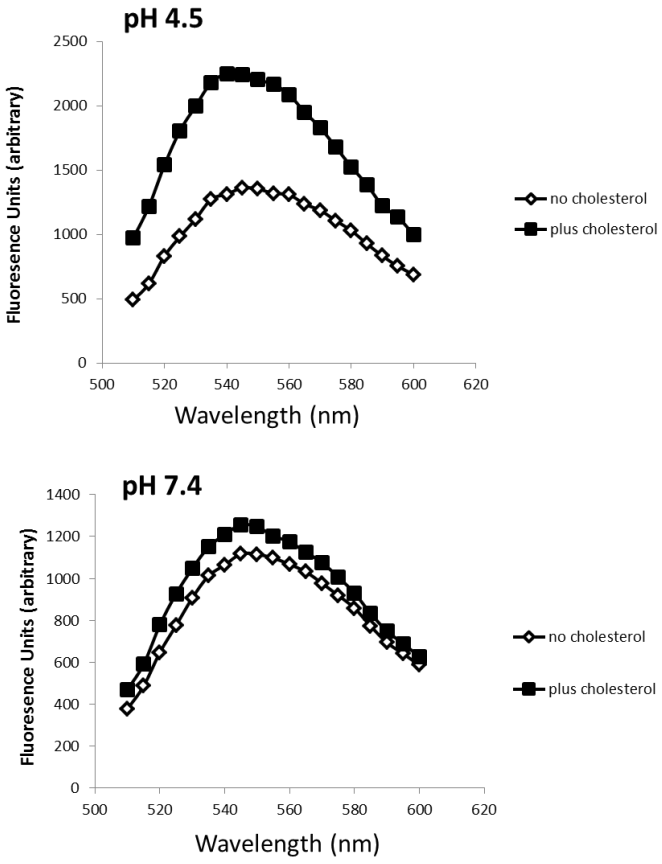


Figure 3.5. CDCs shows a defect in membrane insertion after pH treatment. Membrane insertion was monitored by changes in fluorescence intensity. In a cysteine deficient background(C476A), aspartate 305 was mutated to a cysteine residue (D305C). Recombinant protein was labeled with NBD and fluorescence intensity was measured in the absence of cholesterol and after incubation with cholesterol.

3.3.6 Localization of the pH dependent activity

The crystal structures of several proteins (including PFO, ILY, and ALO)[150-152] have been solved. These proteins have been shown to fold into similar three-dimensional structures, and it is highly likely that the other members of this family also fold into the same four domain structure. Figure 3.6A shows the discontinuous nature of these domains in the primary structure of the protein. In order to localize the pH dependent activity of the protein, chimeric

toxins were created which contained a single domain of INY (or a fragment of a domain) in place of the analogous residues in PLY. These toxins were incubated at the indicated pH for 30min at 37^{o}C before being used in an erythrocyte lysis assay. The chimeric toxin PLY-INYD4 shows a pH dependent activity profile similar to PLY, showing a loss of activity at pH 4.5 (Fig 3.6B). The chimeric toxin PLY-INYD2 also displayed activity consistent with native PLY activity, showing greater hemolytic activity at pH 4.5 than at pH 7.4 (Fig 3.6C). The pH dependent activity of the domain 3 chimeric toxins could not be fully assessed. In a hemolysis assay, these constructs resulted in an "aggregation" phenotype, characterized by red blood cells sticking to the sides of the well plate rather than forming a defined pellet (data not shown). Interestingly, when we examined these red blood cells, we found them to be intact rather than lysed red blood cells. At higher concentrations (Appendix C Fig.C1), this construct was able to lyse red blood cells, but only at pH 4.5 (and not pH 7.4 where the aggregation phenotype was still observed). In order to avoid the aggregation phenotype and assess the role of domain 3 in the pH dependent activity, we created two different chimeric toxins each containing one continuous fragment of domain 3. The INYD3F1 construct contained amino acids 196-245 or domain 3 in a PLY background whereas INYD3F2 contained amino acids 292-366 in a PLY background. Both of these toxins also demonstrated the aggregation phenotype seen for the full length domain 3 chimera (data not shown). These constructs both behaved similarly to the full length domain 3 chimeric toxin, showing hemolytic activity at high concentrations (approximately 1000 fold greater than those needed for native INY) at pH 4.5 but not at pH 7.4 (Appendix C Fig.C2). In our assays, we have observed higher levels of background hemolysis for RBCs incubated at pH 4.5 than for RBCs incubated at pH 7.4 (data not shown). Given the result

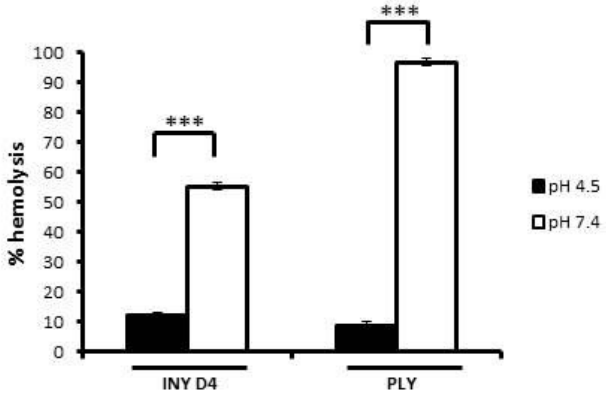
obtained with the chimeric toxins containing INY domain 3 in a PLY background, we attempted to create chimeric toxins with PLY domain 3 fragments in an INY background. We were unable to clone PLY residues 148-197 in an INY background (PLYD3F1). A chimeric toxin composed of PLY residues 244-318 in an INY background (PLY D3F2) showed activity consistent with native PLY losing activity at pH 4.5 (Fig 3.6D). We next assessed the role of domain 1 in the pH dependent activity of the toxin. The chimeric toxin composed of INY residues 1-68 in a PLY background (INYD1F1) displayed the same aggregation phenotype as had been seen with the INY domain 3 chimeric toxins and showed hemolytic activity at pH 4.5, but only at concentrations approximately 1000 fold greater than those needed to achieve the same level of lysis as native INY (Appendix C Fig. 1B). INYD1F2 and INYD1F3 behaved similarly to PLY, showing hemolytic activity at pH 7.4 and losing approximately all hemolytic activity at pH 7.4(Fig 3.6E). We were unable to create a chimeric protein consisting of INY amino acid residues 367-390 in a PLY background. In order to address the role of this fragment (fragment 4) of domain 1 in the pH dependent activity, we created a chimeric toxin of PLY amino acid residues 319-342 in an INY background. This fusion protein behaved similarly to INY, showing approximately 80% lysis at pH 4.5, and dropping to approximately 20% at pH 7.4(Fig 3.6F).

(A)

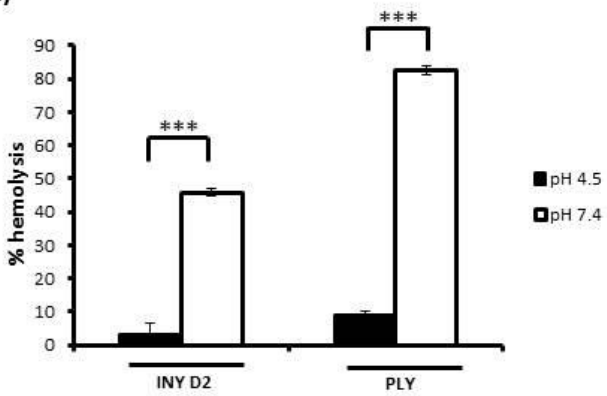
Domains of INY	Residues spanning the domain
Domain 1	1-68, 106-195, 246-291,367-390
Domain 2	69-105, 391-407
Domain 3	196-245, 292-366
Domain 4	408-519

Domains of PLY	Residues spanning the domain
Domain 1	1-21, 58-147, 198-243,319-342
Domain 2	22-57, 343-359
Domain 3	148-197, 244-318
Domain 4	360-471

(B)



(C)



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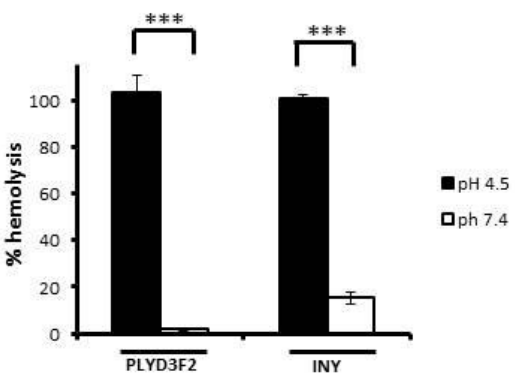

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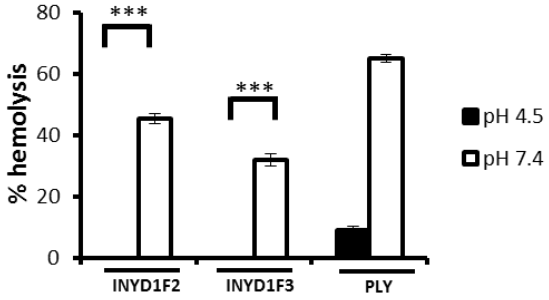

(F)

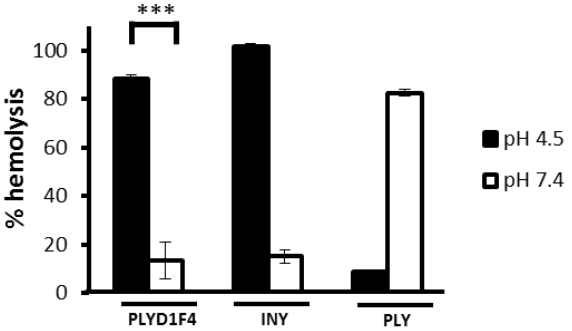

Figure 3.6 Localization of the pH dependent activity. (A) This table shows the amino acid residues spanning each domain. **(B)** INY domain 4 was fused to PLY domain 1-3. Recombinant fusion protein (125 ng/mL) was pretreated for 30 min at 37°C at the indicated pH and used in an hemolysis assay. **(C)** INY domain 2 was fused to PLY D1/3-4. Recombinant fusion protein (125ng/mL) was pretreated for 30 min at 37°C at the indicated pH and used in an hemolysis assay **(E)** INY domain1 fragment 2 and INY domain 1 fragment 3 was fused to the remaining PLY domains. Recombinant fusion protein (125ng/mL) was pretreated for 30 min at 37°C at the indicated pH and used in an hemolysis assay **(F)** PLY domain 1 fragment 4 was fused to the remaining INY domains. Recombinant fusion protein (125ng/mL) was pretreated for 30 min at 37°C at the indicated pH and used in an hemolysis assay .

3.4 Discussion

pH dependent regulation of toxin activity has only been described in a few members of the CDC superfamily: The listerial derived cytolysins (including LLO) as well as PFO. The evolution of this pH dependent activity is consistent with their use in the acidified phagosome to mediate escape into the cytosol. *L. iners* has only been recently identified, and to date no work has been published that suggests that *L. iners* has an intracellular component to its lifestyle. However, its normal physiological niche is the mucosal surface of the vaginal epithelium, which in a normal healthy vaginal tract is maintained at a low pH of about 4. Although some have suggested that most of the CDCs, not including LLO and the listerial derived cytolysins, are pH insensitive[141], our work also suggests that pH dependent activity is a feature of a number of CDCs including pyolysin and inerolysin (which show an acidic pH optimum) as well as pneumolysin and arcanolysin (which show a basic pH optimum). This is suggestive of a pan CDC regulatory mechanism, with various toxins having evolved to operate maximally within the pH range of their anatomical niches.

The molecular mechanisms of LLO pH dependent activity have been localized to a triad of charged residues in domain three which acts as a sensor for environmental pH. Once it has mediated escape from the acidified phagosome, it experiences the more neutral pH of the cytosol, which then initiates a rapid and irreversible unfolding of the TMHs of the portion of the protein which inserts into the membrane, resulting in a complete loss of activity. Interestingly, our results demonstrate the mechanism of pH dependent activity is distinct from that of LLO as it is reversible and affects the very last step of the pore forming mechanism, the insertion of the transmembrane domains, rather than the first step of pore formation, the binding and association with target membranes. Interestingly, higher molecular weight structures were observed for toxin incubated at pH 7.4, supporting the idea that at this pH the toxin is stuck at the stage of oligomerization. It is also possible that toxin treated at this pH forms two types of oligomeric structures, functional prepores as well as higher molecular weight structures which are non-functional. As the concentration of toxin increases, a greater proportion of the oligomers are of this functional type, explaining the ability to overcome this loss of activity with greater amounts of toxin. Our TEM analysis did not show any of these oligomeric strucutres, but our results indicated a different pattern of association with membranes at pH 7.4. The functional significance of this unique association is not clear, but the SDS-AGE results as well as our spectroscopic analysis indicate competence for oligomerization. The pore structures we observed at pH 4.5 were notably larger than any pore structure which has been identified previously. Heterogeneity in pore size has been noted for other toxins[92], but pores this large have never been identified. This result suggests that the pores produced by INY are larger than pores produced by other CDCs. This discrepancy could be resolved through osmotic protection

experiments, getting a rough estimate of the pore size by using dextrans of different molecular sizes to block hemolytic activity of the pores.

Our attempts to localize this pH dependent activity to particular residues were ultimately unsuccessful. However, we know that the residues in domain 3 that confer pH dependent activity on LLO were not sufficient to confer this activity onto INY, confirming a distinct molecular mechanism for the pH dependent activity of INY. The discovery that PLY, which is phylogenetically highly related to INY, displays a loss of activity at neutral pH presented the opportunity to localize the pH dependent activity of INY. Our domain swap approach however was unable to confer PLY pH dependent activity onto INY (and the converse was also true, as we were unable to confer INY pH dependent activity onto PLY). Some of these constructs were not functional in our assays and this is possibly due to incompatibility of INY domains in PLY (as making the converse swaps of PLY domains in an INY background yielded normal activity).

pH dependent activity has been identified in pore forming toxins other than those belonging to CDC superfamily, including diphtheria toxin. For diphtheria toxin, low pH is thought to neutralize acidic loops in the protein, facilitating their insertion into the membrane. Data from PFO studies suggest that at low pH there is a partial unfolding event which primes the protein for membrane insertion[142]. Theoretical calculations of various crystal structures of PFO at neutral or acidic pH suggest that three residues, Asp58, Asp380, and Glu388, function to cause a premature breakage of the link between domain 2 and TMH1/2 of domain 3 at low pH[105]. This explanation would be consistent with our results observed for the domain swaps, indicating the requirement of residues in several domains for this pH dependent activity.

These studies demonstrate that INY has evolved to operate within its particular physiological niche, the relatively acidic vaginal tract. While the molecular basis of this activity has not yet been identified, functionally, INY in the presence of elevated pH shows a defect in the final step of pore formation, prepore to pore conversion. Additionally, our results suggest that the whole family of CDCs are regulated by this subtle mechanism.

Chapter 4: *L. iners* induces unique signaling in the vaginal epithelium

4.1 Introduction

The vaginal epithelium represents the first barrier to pathogen invasion and provides more than just a physical barrier. It possesses an innate immune system which can be selectively activated, allowing it to initiate appropriate responses to pathogenic and commensal species. Via the production of proinflammatory cytokines as well as antimicrobial peptides (defensins), the vaginal epithelium can initiate responses to deal with the vast number of organisms colonizing this surface. The production of these immune system mediators is not only crucial to defense, but has been shown to affect, and perhaps shape, the composition of the colonizing micromicrobiota [153-155].

Although this microbiota is dynamic, complex, and poorly understood the dominant species present in a normal healthy vaginal tract are lactobacilli, with *L. crispatus, L. gasserii,* and *L. jensenii* being the most commonly isolated organisms. Interestingly, the microbiota tends to be dominated by one or two *Lactobacillus* species rather than being a heterogenous mix of species[156]. Several studies have suggested that the presence of *Lactobacillus* species in the vaginal tract prevents the growth of potential pathogens[12]. A loss of these protective species and an overgrowth of these potential pathogens is characteristic of disease states, including bacterial vaginosis (BV). This dysbiosis is associated with significant adverse consequences including an increased risk for preterm birth. The link between preterm labor and BV is supported by epidemiological data but the exact mechanisms remain unclear. Implicated in

this process are IL-8, IL-6, TNFα, as well as the production of prostaglandins and matrix metalloproteases[46], contributing to premature cervical ripening and uterine contractions.[46]

L. iners is a recently identified member of the vaginal tract and epidemiological studies suggest that *L. iners* does not behave like traditionally protective lactobacilli, and rather than contributing to stability is actually associated with increased diversity[122] and transitions into a BV state[66]. We hypothesize that *L. iners*, despite being a *Lactobacillus* species, possesses pathogenic potential and could contribute to BV and BV associated preterm labor. Here, we demonstrate that *L. iners* induces unique signaling in the vaginal tract. Compared to *L. crispatus* (a protective species) and even *G. vaginalis (*a BV associated species), *L. iners* induced responses in the vaginal epithelium, creating an environment characterized by elevated proinflammatory mediators and rich in antimicrobial peptides. We hypothesize that this may contribute to the altered vaginal microbiota seen in BV as well as BV associated preterm labor.

4.2 Materials and Methods

4.2.1 Epithelial Cell Lines

The immortalized human vaginal epithelial cell line VK2/E6E7 was purchased from ATCC (CRL-2616). VK2 cells were cultured in Keratinocyte-SFM (Gibco-BRL) supplemented with 5 ng/mL recombinant epidermal growth factor, 50 µg/mL bovine pituitary extract, 400µM $CaCl_{2,}$10 µg/mL ciprofloxacin at 37°C in a humidified atmosphere with 5% CO_2.

4.2.2 Bacterial Cell Lines

L. iners strains were grown on Columbia agar with 5% sheep blood. *L*. iners type strain DSM 13335, the genome of which has been sequenced, was obtained from the Deutsche Sammlung

von Mikroorganismen und Zellkulturen. *L. iners* strain UPII 143-D(HM-126) and *L. iners* strain UPII 60-B(HM-131) were obtained from BEI resources. *L. iners* liquid cultures were grown in iners media (1% proteose peptone, 1% beef extract, .5% yeast extract, 85.6mM sodium chloride, .830 mM magnesium sulfate, .331mM manganese (II) sulfate, 11.48mM dipotassium phosphate, 2% glucose, 10% Fetal Bovine Serum). *L. crispatus* strains were grown on MRS agar/MRS broth. *L. crispatus* type strain ATCC33820 was obtained from ATCC.

4.2.3 Cloning and expression of mutant CDCs

For the construction of the pore formation deficient mutant, a tryptophan residue at position 481 was replaced with a phenylalanine residue by overlap extension PCR using primers NheI-INY-F(GCCGCCGCTAGCAATACTGAGCCAAAAACAGCTATTG) and INY-TrpPhe-R(*CCACCATTCAAATGCCAAGCCTGTGCATTCTTGAATC*TTAACATTC**AAA**TGACGAACGTTT) and INY-TrpPheF(*AAACGTTCGTCATTTGAATGTTAA*GATTCAAGAATGCACAGGCTTGGCA**TTT**GAATGGTGG) and INY-XhoI-R(GCCGCCCTCGAGTTAGTCATTTTTTACTTCTTCTTTG). Tryptophan to phenylalanine mutation is underlined and in bold. Restriction sites are underlined. Overlaps are underlined and italicized.

4.2.4 Stimulation of cell lines

VK2/E6E7 vaginal epithelial cells were grown to confluence in 12 well plates. Bacteria were heat killed for 15 minutes at 70^{o}C and checked for the absence of live organisms by plating an aliquot onto the appropriate medium. Heat killed bacteria were diluted in keratinocyte-SFM and VK2 cells were treated at an MOI of 10. The plate was spun 39.2 x g for 5 min and then

incubated for 2 hours (to assess the production of proinflammatory cytokines) or 18 hours (to assess for the production of antimicrobial peptides). The amount of LDH released into the cell supernatant was assayed using Cytotoxicity Detection Kit Plus (Roche) as per the manufacturer's instructions. Percent cytotoxicity was determined using the following formula: [(Test LDH-bc)/(hc-bc)] x100 where bc (background control) is an estimate of LDH activity in the assay medium and hc (high control) corresponds to the maximum releasable LDH activity in all cells.

4.2.5 Quantitative real-time PCR

RNA was isolated from cells using an RNAqueous-4PCR (Ambion) according to the manufacturer's instructions. A total of 1 µg of RNA was reverse transcribed into cDNA by using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The quantitative real-time PCR was carried out using power SYBR green master mix in a StepOne Plus thermal cycler (Applied Biosystems). The relative quantification (RQ) values were calculated using a comparative threshold cycle (ΔΔ*CT*) proGram on StepOne software version 2.0.

4.2.6 Statistical Methods

All results are expressed as average values from duplicate readings from one representative experiment. Each experiment was repeated two times. Data were analyzed by Student *t* test. A *P* value of <0.05 was considered significant.

Table 4.1 Primers for proinflammatory cytokine responses

Name	Sequence
Interleukin 8 F	TTGGCAGCCTTCCTGATT TC
Interleukin 8 R	TATGCACTGACATCTAAGTTCTTTAG
GAPDH F	GGGCGCCTGGTCACCAGGGCTG
GAPDH R	GGGGCCATCCACAGTCTTCTG
Interleukin 6 F	AAGAGTAACATGTGTGAAAGC
Interleukin 6 R	CTACTCTCAAATCTGTTCTGG
COX-2 F	TGAGCATCTACGGTTTGCTG
COX-2 R	TGCTTGTCTGGAACAACTGC
Tumor necrosis factor alpha F	TCTCCTTCCTGATCGTGGC
Tumor necrosis factor alpha R	GGTTCAGCCACTGGAGCT

Table 4.2 Primers for antimicrobial responses

Name	Sequence
Human Beta Defensin-2 forward	ATCAGCCATGAGGGTCTTGT
Human Beta Defensin-2 reverse	GAGACCACAGGTGCCAATTT
Human Beta Defensin-3 forward	AGCCTAGCAGCTATGAGGATC
Human Beta Defensin-3 reverse	CTT CGG CAG CAT TTT CGG CCA
Human Beta Defensin-4 forward	ATCAGCCATGAGGGTCTTGT
Human Beta Defensin-4 reverse	GAGACCACAGGTGCCAATTT
Human alpha defensin 5 forward	ATGAGGCTACAACCCAGAAGC
Human alpha defensin 5 reverse	GACTCACGGGTAGCACAACG

4.3 Results

4.3.1 *L. iners* induces proinflammatory signaling in the vaginal epithelium

Epidemiological data as well as genomic studies suggest that *L. iners* is unlike its closely related vaginal counterparts. Many of these studies suggest that *L. iners* has some pathogenic potential, possibly contributing to alterations of the vaginal microbiota or being less protective against disturbances[65, 66]. Others have suggested that *L. iners* could play a role in maintenance or restoration of a healthy vaginal environment[62]. In order to gain further insight into the role of this organism in the vaginal tract and how the vaginal epithelium responds to it (as pathogen or commensal), we treated VK2 vaginal epithelial cells with several heat killed strains of *L. iners, L. crispatus,* and *G. vaginalis.* We found that the *L. iners* strains tested (13335, 60B, 143D) were able to induce significant upregulation of IL-8 (ranging from a 2 fold to 10 fold increase)(Fig 4.1A). All strains tested were able to induce an approximately 6 fold increase in IL-6 transcripts (Fig 4.1B), and induced upregulation of TNFα (ranging from an 6 fold to 10 fold increase)(Fig 4.1C). *L. crispatus* induced no significant production of these targets above the media alone control. Interestingly, we also found that *G. vaginalis*, a BV associated species, did not induce significant production of any of these targets. We also examined the ability of *L. iners* to induce to production of COX-2 (Fig 4.1D). All *L. iners* strains tested were able to induce an approximately six fold increase in COX-2 transcripts. In addition to assessing the level of mRNA transcripts, we also assessed protein levels of IL-8. Here, we found that the one strain of *L. iners* we tested, 13335, induced the production of IL-8 protein in a dose dependent manner. At an MOI of 10, *L. iners* 13335 induced the production of IL-8 (300 pg/mL) after stimulation of VK2 cells for 18 hours (Fig 4.2). In contrast, *L. crispatus* at a similar MOI

induced no significant IL-8 production, in excess of what the untreated cells produced (50 pg/mL). Taken together, these results demonstrate that *L. iners* induces the production of proinflammatory cytokines by the vaginal epithelium, and this not generally shared among lactobacilli.

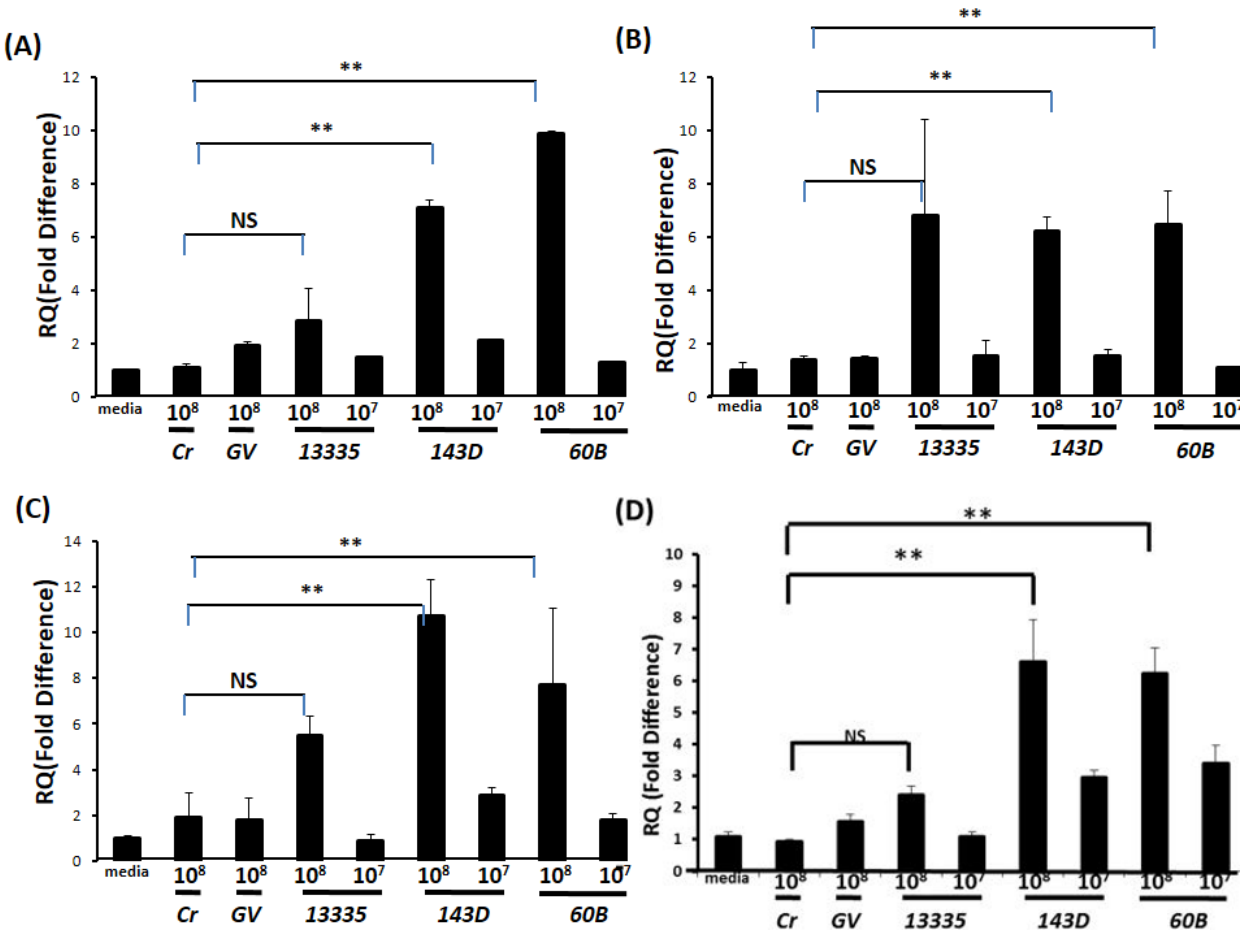

Figure 4.1 Heat killed *L. iners* induces proinflammatory cytokine production by VK2 vaginal epithelial cells. Heat killed bacteria were used to stimulate VK2 vaginal epithelial cells for 2 hours. (A) IL-8 (B) IL-6 (C) TNFα (D) COX-2 production was assessed by RT-PCR. Cell viability was determined by LDH release and for all treatments caused no significant lysis above media alone control. *P values* for this and subsequent figures are as follows * 0.05 **<0.01 ***<0.001

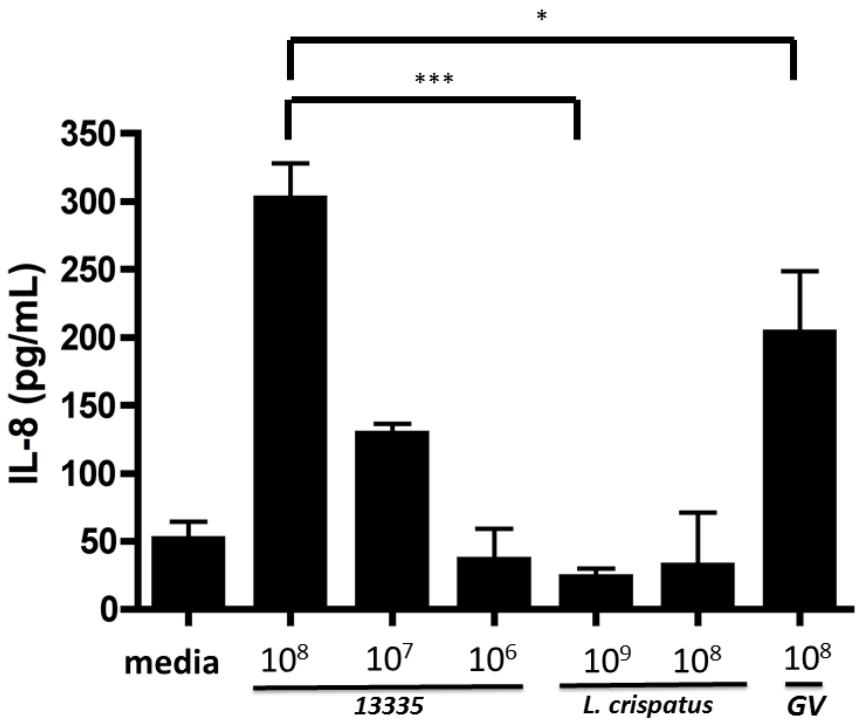

Figure 4.2 Heat killed *L. iners* induces proinflammatory cytokine production by VK2 vaginal epithelial cells. Heat killed bacteria were used to stimulate VK2 vaginal epithelial cells for 18 hours. IL-8 production was assessed by ELISA. Cell viability was determined by LDH release and for all treatments caused no significant lysis above media alone control.

4.3.2 Antibody to CDCs abrogates this proinflammatory cytokine response

We have previously shown that *L. iners* produces INY, a pore forming toxin which belongs to the family of cholesterol dependent cytolysins(Chapter 1 and [143]). This gene is notably missing from the genome sequences of other lactobacilli, including *L. crispatus*. We hypothesized that this pore forming toxin is the main driver for the differential response to these organisms seen previously. Our attempts to develop genetic tools for the manipulation of *L. iners* with the purpose of creating an INY knockout were unsuccessful (Appendix A). To address the role of

this toxin in the responses seen with heat killed bacteria, we utilized a polyclonal antibody raised to a related CDC Vaginolysin (VLY), to neutralize any INY present. Briefly, heat killed bacteria were preincubated with anti-VLY rabbit polyclonal antibody or prebleed (control rabbit serum from before immunization with VLY) before being used to stimulate VK2 vaginal epithelial cells. Heat killed bacteria incubated with prebleed still induced significant production of IL-8 (ranging from a 2 fold to a 30 fold increase in transcripts), IL-6 (ranging from a 2 fold to 10 fold increase in mRNA), TNFα (ranging from a 2 fold to 20 fold increase in mRNA), as well as COX-2 (ranging from 5 fold to 30 fold increase) (Fig 4.3 A,B,C, and D respectively). Pre-incubation with anti-VLY antibody abrogated the production of these targets to levels comparable to untreated cells.

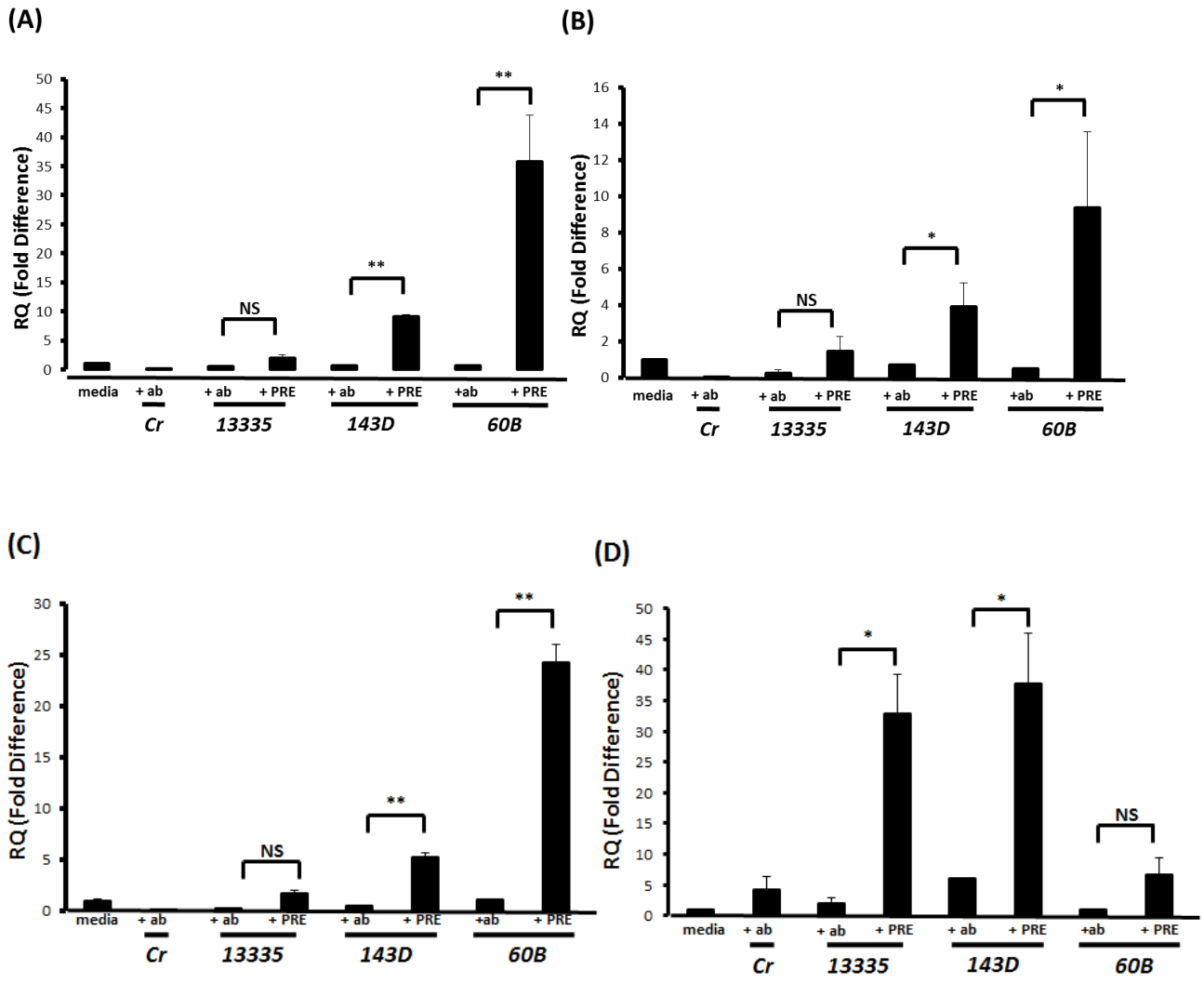

Figure 4.3 Antibody to CDCs abrogates the proinflammatory cytokine response . Heat killed bacteria were incubated with anti-VLY or prebleed at 1:20 for 20 minutes and then used to stimulate VK2 vaginal epithelial cells for 2 hours. (A) IL-8 (B) IL-6 (C) TNFα and (D) COX-2 production was assessed by RT-PCR. Cell viability was determined by LDH release and for all treatments caused no significant lysis above media alone control.

4.3.3 Purified Recombinant INY is sufficient for the proinflammatory cytokine response

To further assess the role of the toxin in these responses, we wanted to determine if purified recombinant toxin alone was sufficient to induce the production of these proinflammatory

cytokines. VK2 vaginal epithelial cells were treated with purified recombinant toxin alone or a mixture of toxin and heat killed *L. crispatus*. Recombinant INY was able to induce significant upregulation of IL-8, IL-6, TNFα, and COX2 in a dose dependent manner. An approximately 100 fold increase in IL-8(Fig 4.4A), 90 fold increase in IL6 (Fig 4.4B), 25 fold increase in TNFα (Fig 4.4C), and 10 fold increase in COX-2 (Fig 4.4D) was observed after stimulation with purified recombinant INY. The addition of *L. crispatus* had no effect on the production of these targets.

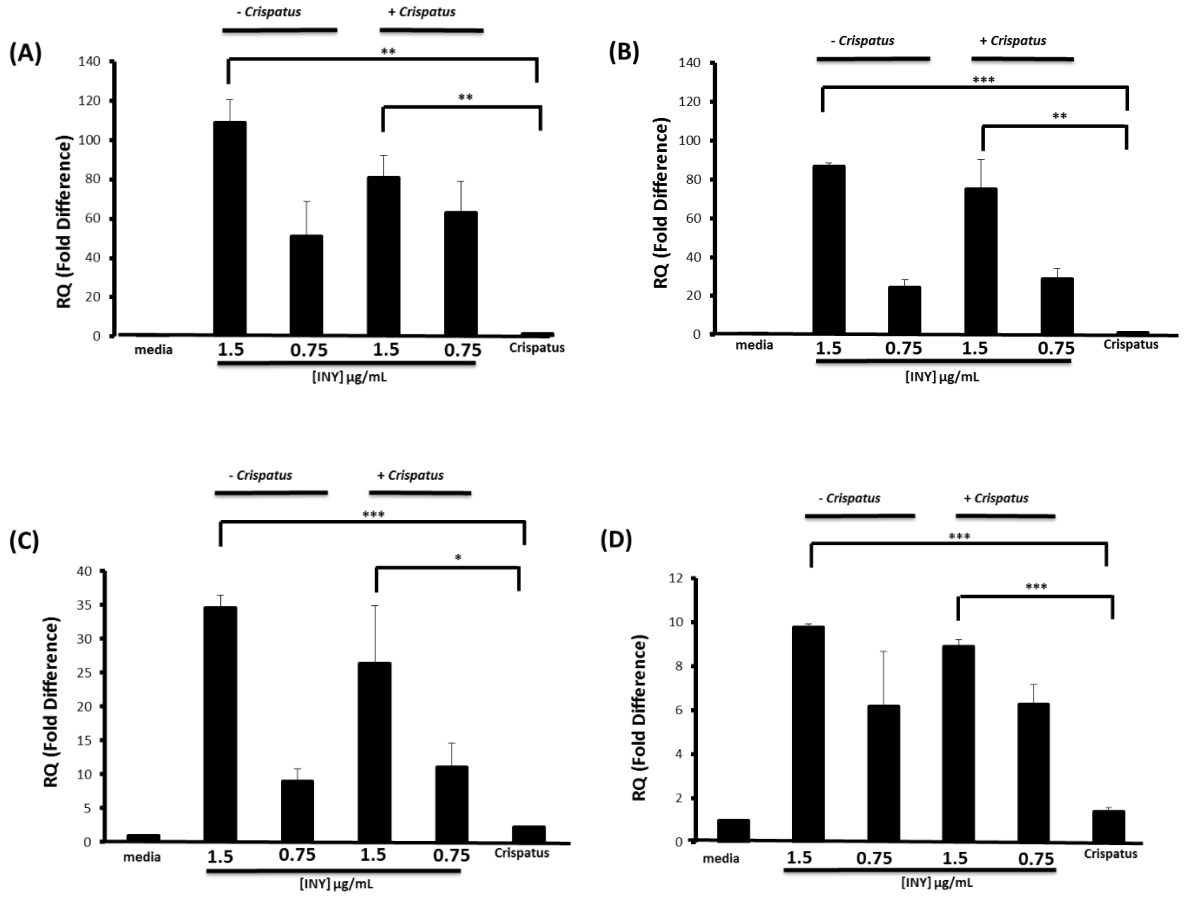

Figure 4.4 Recombinant INY can stimulate proinflammatory cytokine production. Recombinant INY was used to stimulate VK2 vaginal epithelial cells for 2 hours. (A) IL-8 (B) IL-6 (C) TNFα and (D) COX-2 production was assessed by RT-PCR. Cell viability was determined by LDH release and for all treatments caused no significant lysis above media alone control.

4.3.4 Pore formation is required for proinflammatory cytokine production

TLRs play an important role in recognizing microbial infections and initiating responses, usually resulting in the elaboration of several proinflammatory cytokines. Previous work has demonstrated that CDCs can act as more than just cytolytic toxins, and can induce proinflammatory signaling via direct recognition by TLR4[157, 158], induction of osmotic stress[134], as well as allowing the introduction of bacterial products into the cytosol of the cell and sensing by intracellular pattern recognition receptors[109]. We wanted to determine if the responses we observed were due to direct recognition of toxin or pore formation. To address this, a point mutant toxoid (W481F) was created which was 1000 fold less efficient than wild type toxin at forming pores (Fig 4.5A). We found that this mutant, although still able to bind to the surface of red blood cells at comparable levels (Fig. 4.5B) was unable to induce any significant production of IL-8 (Fig. 4.5C), IL-6 (Fig. 4.5D), TNFα (Fig. 4.5E), COX-2 (Fig. 4.5F).

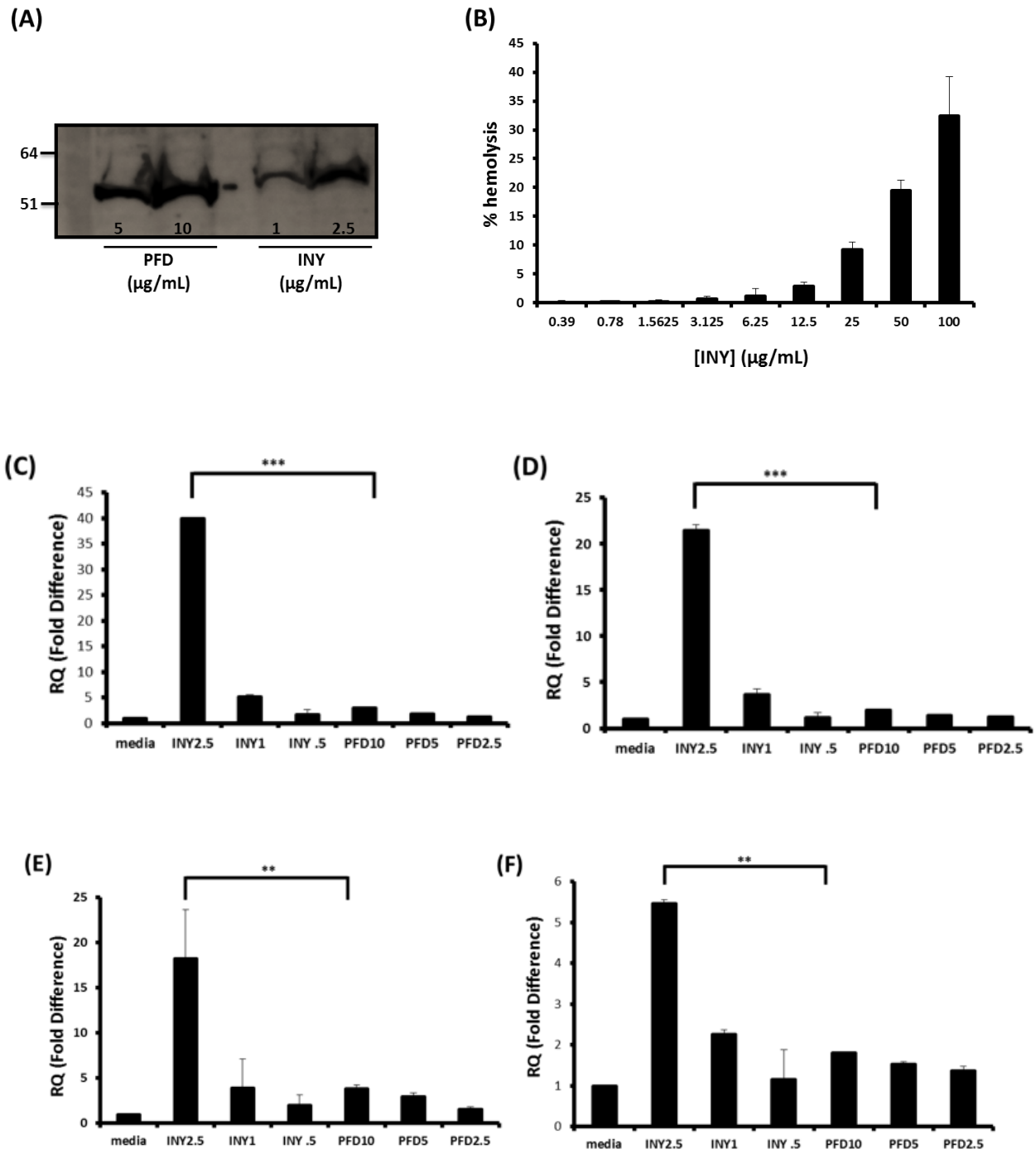

Figure 4.5 Pore formation is required for proinflammatory cytokine production. Recombinant INY or a pore formation deficient mutant, PFD (W481F) were (A) Incubated with human erythrocytes and binding was assayed by western blot (B) used in a hemolysis assay. Recombinant toxins (µg/mL) were used to stimulate VK2 vaginal epithelial cells for 2 hours. (C)IL-8 (D) IL-6 (E) TNFα and (F)COX-2 production was assessed by RT-PCR. Cell viability was determined by LDH release and for all treatments caused no significant lysis above media alone control

4.3.5 Heat killed *L. iners* induces a unique defensin profile

The vaginal epithelium must discriminate between pathogen and commensal, initiating unique responses to each. In addition to the production of proinflammatory cytokines, vaginal epithelial cells may also produce an array of antimicrobial peptides, and in other anatomical sites it has been suggested that these substances play a critical role in regulating the composition of the microbial microbiota[159]. We examined the defensin profile induced by *L. iners* and *L. crispatus* and found that *L. iners* stimulation of VK2 cells resulted in a 2 fold to 3.5 fold increase in HBD4 (Fig. 4.6A). In response to stimulation with *L. iners* strain 60B, we observed a 2.5 fold increase in HD5 and in response to *L. iners* 13335 stimulation we observed an approximately 20 fold increase in HD5 (Fig. 4.6B), Both strains tested induced an approximately 4-5 fold increase in HBD2 (Fig. 4.6C). *L. iners* was not able to stimulate HBD3 production, but we did note a downregulation of HBD3 by *L. crispatus,* although this was not statistically significant. When purified recombinant INY was used, we did not see comparable induction of defensins as with whole heat killed bacteria. Our results demonstrate that *L. iners* has the ability to induce responses in the vaginal epithelium, creating a unique vaginal environment, as compared to *L. crispatus*, a traditionally protective species. We hypothesize that this unique complement of defensins has important implications for the composition of the colonizing micromicrobiota.

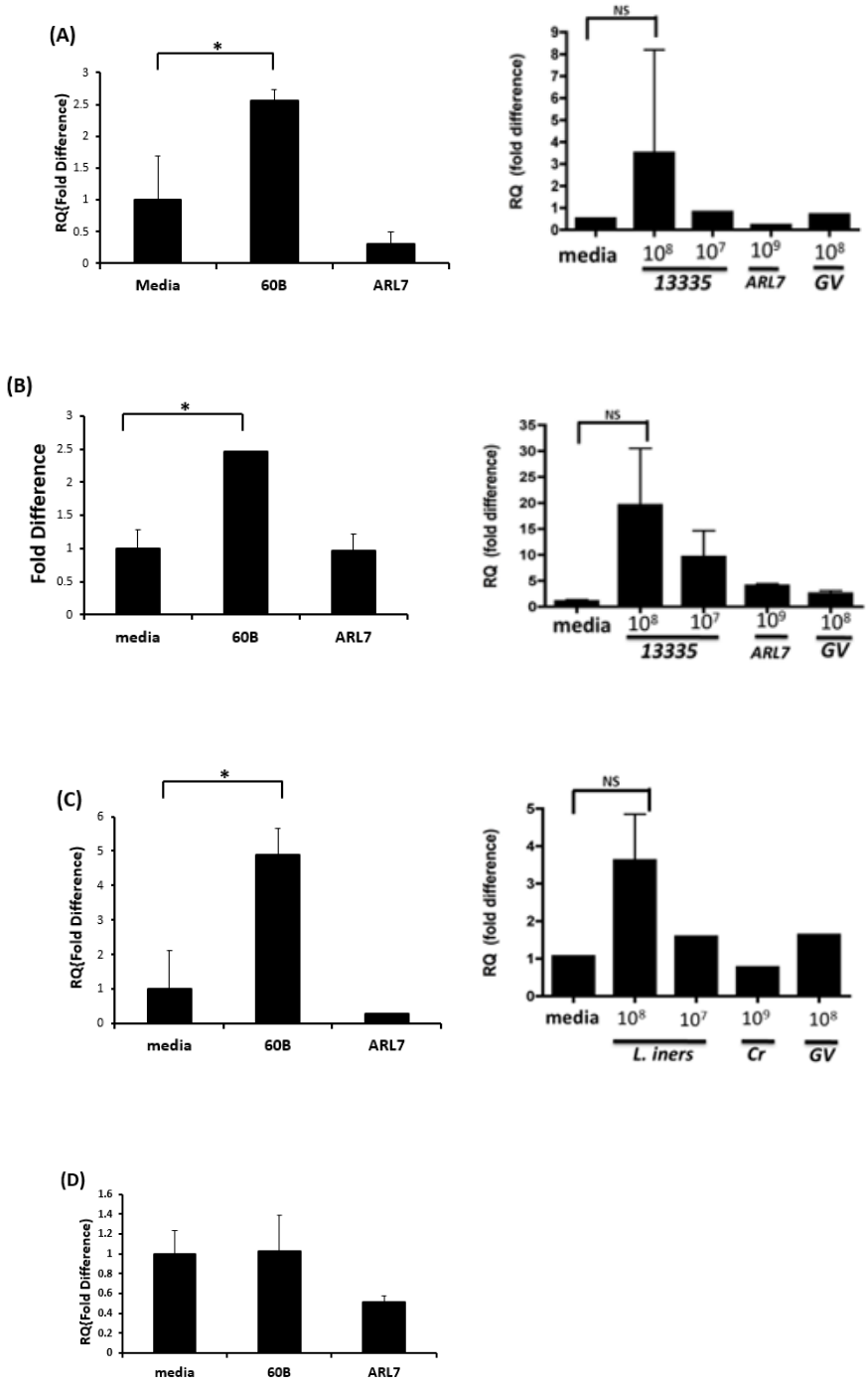

Figure 4.6 Heat killed *L. iners* induces unique defensin production by VK2 vaginal epithelial cells. Heat killed bacteria were used to stimulate VK2 vaginal epithelial cells for 18 hours. (A)HBD4 (B) HD5 (C) HBD2 and (D)HBD3 production was assessed by RT-PCR. Cell viability was determined by LDH release and for all treatments caused no significant lysis above media alone control

4.4 Discussion

Traditionally, a healthy vaginal tract is defined by a microbiota which is dominated by *Lactobacillus* species. As culture independent methods for the identification of bacterial species has become more commonplace, our understanding of the composition of a normal vaginal tract has become more complete. The most commonly isolated *Lactobacillus* strains are *L. crispatus, L. gasseri,* and *L. johnsonii* and the use of these culture independent techniques have expanded the group of vaginal colonizing bacteria to include a new *Lactobacillus* species, *L. iners*. Ravel et. al demonstrated the ubiquitous nature of this organism, with 30% of his study population being dominated by this organism. Vaginal lactobacilli are thought to prevent the growth of urogenital pathogens via the elaboration of lactic acid, hydrogen peroxide and bacteriocins. Interestingly, *L. iners* displays paradoxical behavior as it is associated with increased transitions into BV states as well as showing a vaginal environment characterized by increased diversity and more elevated pH as compared to an *L. crispatus* dominated microbiota[122]. The idea that this organism possesses pathogenic potential is further supported with the identification of the first putative virulence factor, inerolysin, a pore forming toxin belonging to the family of cholesterol dependent cytolysins[143]. We sought to understand what the vaginal environment would look like for an *L. iners* dominated microbiota, and how this differed from a microbiota dominated by a traditionally protective *Lactobacillus* species, *L. crispatus*.

The vaginal epithelium is the initial point of contact between a microorganism and the host. This epithelium must be able to respond to commensal and pathogen and initiate appropriate responses to each. Commensals like *L. crispatus* and *L. jensenii* have been shown to be fairly innocuous, initiating no significant proinflammatory signaling in the vaginal epithelium, whereas pathogenic species upregulate these targets[160]. Furthermore, these commensals have been shown to actually downregulate proinflammatory signaling after TLR stimulation[161]. Interestingly, we found that *L. iners* rather than behaving like a commensal lactobacilli, actually induced responses more characteristic of a pathogenic species characterized by increased production of IL-8, IL-6 and TNFα. Additionally, we also looked at the induction of cyclooxygenase-2 which is responsible for the production of prostaglandins and found that *L. iners* strains also upregulated this target as well. Excessive proinflammatory cytokine production can be harmful to a pregnancy and contribute to preterm labor[162-165]. While the roles of these cytokines in preterm labor have not been fully elucidated, previous work demonstrates that IL-8 can induce neutrophil influx into the cervix where these immune cells can the produce MMP-8 (neutrophil collagenase) which can contribute to cervical ripening. TNFα can also contribute to cervical ripening and preterm labor via the induction of MMP-8, MMP-9[166]. IL-6 has been used as a biomarker for the prediction of preterm labor, and it is suggested that this inflammatory mediator could induce the upregulation of oxytocin receptor expression in the myometrium[167], which could contribute to uterine contractions. It is also known that prostaglandin production by COX-2 can result in increased calcium concentrations in the myometrium which could contribute to uterine contractions. Most of these studies have examined cytokines and neutrophil influx in the cervix, uterus, and maternal and fetal

membranes. However, it is very likely that inflammatory mediators produced by the vaginal epithelium could ascend to induce changes further along the vaginal tract.

Our results suggest that the main driver for the differential response of vaginal lactobacilli (*L. iners* vs. *L. crispatus*) is INY. After neutralization of INY by an antibody raised to a related CDC, the responses we observed were completely abrogated. Epithelial cells can detect the presence of toxins either via its pore forming abilities in response to calcium fluxes[134] or as a result of introduction of bacterial cell components through the pore[109]. Our results suggest that the induction of these targets is accomplished through calcium fluxes, as we used purified recombinant protein to induce these responses, in the absence of any additional bacterial components. It is possible that our preparations of purified recombinant protein contain some contaminants which could enter the cytosol via the pore to induce these proinflammatory responses. Further work examining the requirement of NOD receptors could shed light on the importance of translocation of bacterial components for the initiation of these responses. Additionally, CDCs could induce these responses by direct recognition of toxin via TLR4[157]. We show that pore formation, and not direct recognition is responsible for the responses we observed, as INY toxoid (INYW481F) was unable to induce these responses despite similar membrane binding activity.

In addition to providing a physical barrier, the vaginal epithelium can antagonize the growth of potential pathogens via the production of defensins. These antimicrobial peptides have the ability to directly inhibit the growth of microorganisms, as well as more complex effects linking the innate and adaptive immune system. Additionally, it has been demonstrated that these

antimicrobial peptides can shape the composition of the microbiota[155, 168]. We demonstrate that *L. iners* induces the production of a unique profile of defensins, as compared to *L. crispatus* which did not induce any of the targets we assessed and actually seemed to downregulate them. In a study of a pregnant population, vaginal fluid from women who had an intermediate microbiota (which is thought to be dominated by *L.* iners) was enriched in defensins, as compared to normal microbiota as well as women with frank bacterial vaginosis[169]. Interestingly, the production of INY by this strain is not what drives this response, and requires some unknown stimulating factor. We suggest that a *L. iners* dominated microbiota, via the production of several antimicrobial targets can contribute to alterations and potentially dysbiosis of the vaginal micromicrobiota, resulting in a transition to a BV state and a reduction in the level of defensins. More work is required to understand the full effect that production of these defensins have on health and disease in the vaginal tract.

We present evidence that clearly demonstrates that *L. iners* and *L. crispatus* induce unique responses in the vaginal epithelium. These data suggests that the vaginal environment of an *L. iners* dominated microbiota is distinct from that of an *L. crispatus* domainted microbiota, characterized by increased proinflammatory cytokine production and a unique profile of defensins. This altered defensin profile potentially contributes to the dysbiosis seen in bacterial vaginosis, shaping the micromicrobiota in a way that is distinct from the way in which an *L. crispatus* dominated microbiota. Attempts to treat bacterial vaginosis (a state not rich in proinflammatory mediators) and limit preterm labor have been unsuccessful, suggesting that the events contributing to preterm labor have set in long before we ever see an episode of BV. Given the ability of *L. iners* to persist in both “normal” and BV states, the chronic elevated

proinflammatory cytokine profile could potentially contribute to BV associated preterm labor. Figure 4.7 outlines the role *L.* iners may play in vaginal health. The finding that INY is important in these responses presents a new target for limiting the incidence of preterm labor. Further study is required to determine if these responses, in vivo, could result in alterations of the microbiota and increase the risk of preterm labor.

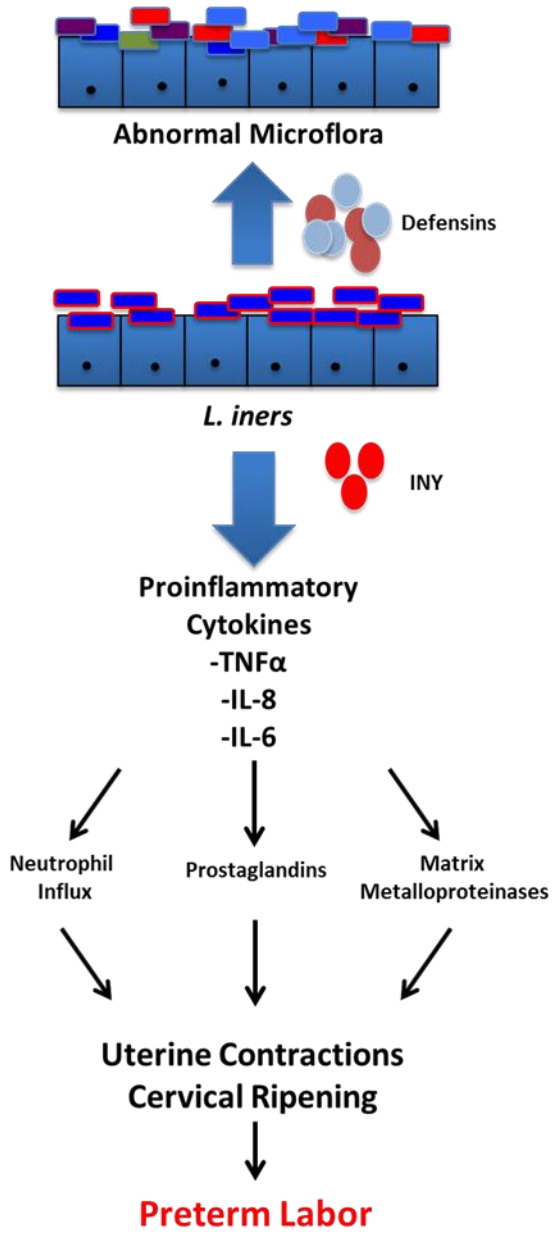

Figure 4.7 Proposed model for the role of *L. iners* in the vaginal tract. In an *L. iners* dominated flora, the vaginal environment is enriched in proinflammatory cytokines as well as unique profile of defensins. We hypothesize that via the production of this unique defensin profile, *L. iners* initiates changes in the microbial flora (or is less able to stabilize the flora after some insult) contributing to the onset of bacterial vaginosis. Additionally, before an episode of BV ever set in, there is a chronic production of proinflammatory cytokines. Via the production of these inflammaory mediators, *L. iners* contributes to the processes implicated in preterm labor.

Chapter 5: Summary

5.1 Summary of Results

L. iners is a recently identified constituent of the vaginal tract. Initially overlooked because of its fastidious nature, the use of cultivation independent techniques for identification allowed for the expansion of the repertoire of *Lactobacillus* species colonizing the vaginal tract to include this organism. Traditionally, lactobacilli have been ascribed protective and stabilizing effects, contributing to the overall health of the vaginal tract[12, 13]. Techniques for the molecular identification of bacterial species have led to the understanding that the lactobacilli colonizing the vaginal tract are not a single species, but rather a group of distinct species including *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners*. Epidemiological studies have furthered our knowledge about the role of these organisms in the vaginal tract, and have suggested that attributing a protective function to these species as a whole may not accurately reflect the role they play in shaping the vaginal microbiota and determining the health of the vaginal tract[9, 121]. In particular, *L. iners* was found to be associated with increased transitions into BV states while *L. crispatus* was not, suggesting that *L. iners* has pathogenic potential. It is this idea which led us to look for putative virulence factors in the genome of this organism. Our results demonstrate that *L. iners* produces a pore forming toxin which belongs to the cholesterol dependent cytolysin (CDC) superfamily. This family of toxins is secreted by a wide range of Gram positive and Gram negative organisms, and play important roles in the pathogenic mechanisms of the bacteria which secrete them. The three dimensional structure of several CDCs have been solved, and across the family there is a high level of sequence identity and similarity, suggesting that this family of toxins possesses similar three-dimensional structure

and thus function in similar ways[105, 152, 170]. As their name suggests, these toxins are dependent on cholesterol to serve as the initial receptor mediating pore formation. While these varied toxins all act through the same mechanism, pore formation, their activity may be required at particular times during the life cycle of the organism. Regulation may occur at the level of transcription, translation, or post-translationally. In our review of the literature, there has been only a single report of regulation at the level of transcription, demonstrating that the CDC produced by *B. anthracis* was regulated by external conditions[116]. Greater evidence for regulation at the level of activity has been shown for both LLO and PFO, showing optimal activity at acidic pH[102, 142]. The molecular basis for this activity has been identified in LLO, but not PFO[103]. Furthermore, no other groups have demonstrated pH dependent activity for any other members of this toxin family. Given that this organism occupies a normally acidic nice, we hypothesized that this toxin (like LLO and PFO) would show an acidic pH optimum and that this activity was a common mechanism for regulation among many members of the class of cholesterol dependent cytolysin.

Additionally, we sought to explain the paradoxical behavior of *L. iners* among lactobacilli, demonstrating increased transitions of the vaginal microbiota in women with a microbiota dominated by this species. Our work here demonstrates that *L. iners* induces unique signaling in the vaginal epithelium which we hypothesize contributes to its paradoxical behavior. These findings are discussed further in the following sections.

5.2 Identification and Characterization of the Cholesterol Dependent Cytolysin produced by *L. iners*

L. iners is an atypical organism which has been recently identified due to the use of culture independent methods for identification of microbial species. Epidemiological studies suggest that this organism behaves more like a pathogen than a protective lactobacillus species, demonstrating reduced stability of the vaginal microbiota in individuals colonized by *L. iners* as compared to *L. crispatus.* This paradoxical behavior led us to look for toxins resembling vaginolysin (produced by *G. vaginalis*, a potential BV associated pathogen) in the genome of this organism. BLAST analysis resulted in the identification of an ORF with a high similarity to vaginolysin in all of the sequenced *L. iners* strains available on NCBI. Interestingly, the presence of this putative toxin was unique to *L. iners* strains and not present in any of the other vaginal lactobacillus species for which fully sequenced genomes were available. This toxin was named Inerolysin (INY). This is the first report of INY being purified, and we sought to characterize it. Recombinant INY displayed non-species restricted activity that displayed characteristics common to the family of cholesterol dependent cytolysin superfamily: activated by DTT and inhibited by the addition of exogenous cholesterol. Additionally, antibody raised to a related family member was able to recognize, and neutralize INY. Our results expand the family of cholesterol dependent cytolysins to include the pore forming toxin produced by *L. iners*, referred to as INY. The identification of this putative virulence factor began to lend support to the idea that *L. iners* possessed pathogenic potential and could contribute to disease in the vaginal tract. The expansion of this family of toxins provides further opportunity to study the

structure-function relationship of the CDC superfamily as well as further our knowledge of the mechanism of pore formation.

5.3 Characterization of the pH dependent activity of INY

Others have demonstrated that CDCs produced by organisms with an intracellular component to their lifecycle, have evolved to be well suited to the acidic pH of the phagosome. Although it has not been shown that *L. iners* has an intracellular lifestyle, it does normally occupy the acidified vaginal tract (pH 4.0) and so we sought to determine if INY demonstrated pH dependent regulation similar to PFO and LLO. Our results demonstrated that the acidic pH optimum we see for INY is distinct from LLO, as it is reversible in INY, and LLO like activity could not be conferred onto INY by just the addition of the analogous "sensor" residues which are responsible for the pH dependence of LLO. We determined that membrane binding, and oligomerization could proceed, but the final step in pore formation is defective at more neutral pH. Interestingly, pH dependent activity was not restricted to INY, but was also identified in pyolysin and arcanolysin, phyologenetically distant relatives of INY. While most of the toxins that have been examined and in work from other groups as well as our work examining INY and PYO show optimal activity at acidic pH, this is the first report of toxins which exhibit optimal activity at more neutral pH. Interestingly, those toxins which demonstrate acidic pHs occupy an acidic niche during their lifestyle. In the case of *Listeria* and *Clostridia,* an acidic pH optimum is required for activity in the acidified phagosome. Those which demonstrate more neutral pH have not been shown to occupy any intracellular or acidified niches, and remain mainly extracellular where the pH is normally more neutral. Taken together, past work and the studies carried out in this work suggests that pH dependent regulation of toxin activity is a mechanism

common to the entire family and that each toxin may be particularly well suited to its physiological niche. Further work is required to confirm that all CDCs have pH dependent activity. Analysis of pneumolysin suggests that the mechanism of PLY pH dependent activity is the same as it is for INY, as membrane binding and oligomerization were intact at neutral pH. We did not directly test for transmembrane helix insertion spectroscopically as we did with INY, but it could be done in the future to confirm that the defect is at this stage of pore formation. Our attempts to localize the pH dependent activity were ultimately unsuccessful, but suggest that the pH dependent activity is a function of several domains, rather than just a single one as it is with LLO. INY is well suited to its physiological niche, colonizing the acidified vaginal tract. INY shows optimal activity at acidic pH and so may be able to initiate changes in the normal acidified vaginal tract. *G. vaginalis*, another organism which makes a CDC, is a potential vaginal pathogen whose exact role in BV has not yet been elucidated. While it is possible that this toxin may play a role in contributing to disease and adverse consequences associated with BV, the fact that VLY has optimal activity at neutral pH argues against its role in initiating changes in the normally acidified vaginal tract. Additionally, *G. vaginalis* and its associated toxin are probably not associated with initiating changes in the vaginal microbiota as these organisms are rarely found in large numbers in the normally acidified vaginal tract. We suggest that INY is particularly well suited to acting on the vaginal mucosa when it is in a "normal" state and is acidified. Through some unknown mechanisms (investigated in Chapter 3), this toxin may act to alter the vaginal microbiota, contribute to transitions into BV states, as well as contribute to BV associated adverse outcomes. Additionally, the work carried out in this study furthers our knowledge of the ways in which CDC activity can be regulated and suggests that the entire

family of CDCs may be regulated by pH. Further studies must be carried out to determine the molecular basis of this pH dependent regulation.

5.4 Characterization of the responses of the vaginal epithelium to *L. iners*

Given the paradoxical behavior of this organism, and its potential contribution to mediating transitions into BV states, we wanted to look at the responses of the vaginal epithelium to this organism in comparison to a traditionally protective *Lactobacillus* species. Our results demonstrate that *L. iners* behaves more like a pathogenic species, inducing the production of proinflammatory cytokines IL-8, IL-6, and TNFα. Additionally, we hypothesized that this organism could be a major factor in the etiology of BV associated preterm labor, given its ability to persist in both normal and BV states. We found that *L. iners* induced significant production of COX-2, which plays a large role in preterm labor (in addition to the proinflammatory cytokines mentioned previously). The main driver for this response was found to be INY, and this response required active pore formation. Further work is required to clearly demonstrate a link between *L. iners* and preterm birth, but if INY does contribute to this adverse consequence it presents a new target for limiting the incidence of BV associated preterm labor. Our results also demonstrate that *L. iners* induces the production of a unique profile of defensins in the vaginal tract. We speculate that this unique signaling could contribute to instability and increased frequency of transition into a BV state associated with *L. iners* colonization. Further work is required to understand how this complement of defensins translates to actual changes in the composition of the microbial microbiota. While we were unable to accomplish this,

mouse models of *L. iners* colonization could yield a significant amount of information with regards to the vaginal environment dominated by *L. iners.* Given the non-species specific nature of this toxin, there is no reason to suspect that *L. iners* could not colonize the vaginal mucosa of the mouse. However, one potential problem with this model is that the vaginal tract of the mouse is more basic than the human vagina[171], which could antagonize both the colonization by this organism as well as the activity of this toxin. Additionally, purified toxin could be utilized in a mouse model to understand how this CDC may alter signaling in the vaginal mucosa. Overall, the vaginal epithelium recognizes *L. crispatus* and *L. iners* in very different ways (mediated in part by INY) and induces very unique responses to each of them. This suggests that the presence of *Lactobacillus* species is not necessarily synonymous with vaginal health. Current methods for diagnosis of BV depend on clinical criteria, such as Gram stain, whiff test, presence of discharge as well as clue cells. These methods can detect the presence of BV in symptomatic women, but a significant number of women are asymptomatic. Better techniques are required to identify these asymptomatic women. The work carried out here suggests that women who are preferentially colonized by *L. iners* may still be at risk for some of the adverse outcomes associated with BV, such as preterm labor. This should prompt us to develop new tools to identify those women who are preferentially colonized by *L. iners* and thus at risk for BV and BV associated preterm labor.

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Appendix A: Attempts to generate an INY knockout

A1.1 Introduction

Lactic acid bacteria are a group of organisms which are Gram-positive, non-spore forming bacteria, initially isolated from milk[172], and can be found in fermented food and even the human body. This group of organisms represents a major constituent of the gastrointestinal tract, where they function in host metabolism as well as protection against pathogen invasion/growth. Given their crucial roles in the food industry as well as the human body as well as the fact that they are generally regarded as safe (GRAS), there has been significant interest in the genetic manipulation of these organisms. Much of the interest has been in the realm of food development, being use for improved food preservation as well as flavoring[173]. One of the more medically relevant uses is for improved vaccine delivery. These organisms provide attractive vehicles as they can be delivered orally (which can enhance adherence) and they can induce both mucosal and systemic immune responses. As such, there is a significant amount of work that has been done on generating tools for genetic manipulation as well as techniques for transformation of these organisms.

The development of *lactobacillus* plasmids and vectors has been ongoing for quite some time and great strides have been made in developing vectors which can be utilized for cloning. Plasmids for use in these organisms can be broadly categorized as those which are promiscuous (and thus able to replicate in several organisms), those with two origins of replications (which facilitates manipulation in *E. coli* followed by transformation of lactobacilli), and native *Lactobacillus* plasmids engineered with a Gram negative origin of replication[174]. In our studies, we made use of several of these plasmids to optimize the conditions for the introduction of foreign DNA into this previously untransformed *Lactobacillus* species.

In order to generate INY knockouts to study the role of this toxin in disease processes, we made use of integration vectors. There has been a significant amount of work done to generate new integration vectors based on the presence of insertion sequences present normally in lactobacilli[175]. The efficiency of a number of these vectors has been improved by engineering plasmids with temperature sensitive origins of replication. These plasmids should replicate at permissive temperatures, which should facilitate recovery of transformants even if our transformation efficiency is low. These transformants can then be shifted to a higher non-permissive temperature which will facilitate integration. We created several integration plasmids with a truncated form of INY to direct the integration events. In the presence of antibiotic selection only those cells which have undergone the first crossover event and thus have integrated the plasmid should be resistant to that antibiotic. These transformants would then be grown for a second time in the absence of selection to stimulate a second crossover event, resulting in a looping out of the original plasmid elements, and leaving either the full length gene or the truncated version of the gene in the genome. In either case (either a single crossover event or a double crossover event), we would have a functional INY knockout. However, the first situation could potentially have polar effects, affecting the expression of other genes upstream and downstream. The second scenario would have created a knockout with no possibility of polar effects. Figure A1 shows a schematic of the proposed crossover events that would occur.

The other major modifiable parameter we manipulated in order to generate a genetically modified organism was the mode by which DNA was introduced. Electroporation has been widely used in lactobacilli, and acceptable transformation frequencies have been demonstrated for a number of lactobacillus species including *L. gasseri* and *L. johnsonii[174, 176, 177].* Additionally, conjugation provides an alternative means for the introduction of foreign DNA into host cells. This form of horizontal gene transfer has been demonstrated between lactobacillus species[178, 179] but also between *Lactococcus* and *Lactobacillus* [180]. In our study, we made use of the pAMβ1 plasmid which is

capable of self-transmission. This plasmid has been used in the past to transmit genes encoding clumping factors to the host cell, and we hoped to utilize this same technique for the introduction of foreign DNA. This would be the first report of genetic manipulation of *L. iners* and would facilitate further work in understanding the role of this organism in health and disease of the vaginal tract.

A1.2 Materials

A1.2.1 Growth media and buffers

Iners Media

1% proteose peptone, 1% beef extract, .5% yeast extract, 85.6mM sodium chloride, .830 mM magnesium sulfate, .331mM manganese (II) sulfate, 11.48mM dipotassium phosphate, 2% glucose, 10% Fetal Bovine Serum

Electroporation Buffer1

0.4M sucrose, 1mM $MgCl_2$, 5mM KH_2PO_4 pH 6.0

Electroporation Buffer 2

0.9M sucrose, 3mM $MgCl_2$

Electroporation Buffer 3

0.5M sucrose, 10% glycerol

Electroporation Buffer 4

0.5M Sucrose, 7mM K_3PO_4, 1mM $MgCl_2$, pH 7.4

A1.2.2 Bacterial strains and plasmids

Strains/Plasmid	Relevant Characteristics	Reference or source
E. coli		
TOP10	F mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 deoR recA1 araD139 (ara-leu)7697 galU galK	Invitrogen
TG1	K-12 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^-m_K^-$)	Stratagene
ARE165	NEB10+pRK21761; contains RK2 helper plasmid for conjugal transfer	[181]
L. iners		
13335	Type strain; isolated from human urine	DSMZ
60B	isolated from human vagina	ATCC
143D	isolated from human vagina	ATCC
Plasmids		
pCR2.1-TOPO	Cloning vector; Amp^R Km^R	Invitrogen
pARE252	1.2kb INY fragment consisting of the first 507 base pairs fused to the last 479 base pairs and lacking the internal 300bp	This work
pAM1	E. coli-Bifidobacteria shuttle vector; Amp^R, Erm^R	[182]
pARE253	Truncated INY from pARE252 subcloned into XbaI/HindIII site of pAM1	This work
pEVP3	pneumococcal suicide vector; Cm^R	[183]
pARE254	Truncated INY from pARE252 subcloned into KpnI/XbaI site of pEVP3	This work
pGID023	Integration vector; Erm^R	[184]
pARE298	Truncated INY from pARE252 subcloned into the BamHI/XbaI site of pGID023	This work
pJAK16	Cloning vector; Cm^R	[185]
pG+Host9	broad host range temperature sensitive plasmid; Erm^R	[186]
pARE284	Truncated INY from pARE252 subclones into the EcoRI site	This work
pRK21761	RK2 helper plasmid for transfer via conjugation	[156]
pOri23	Expression vector for Gram positive; ErmR	[187]
pKS80	Expression vector for Gram positive; ErmR	[188]

A1.3 Procedures

A1.3.1 Preparation of electrompetent cells

L. iners was grown in iners media for approximately 26 hours (which corresponds to log phase) at 37°C/5% CO_2. Iners media was also supplemented with sodium chloride (0.9M and 0.5M)[189] as well as glycine (0.8%, 1%, 2%, 2.5%). No growth was observed in media supplemented with 0.9M NaCl and 2.5% glycine. These agents are known to destabilize the cell wall. Cells were harvested and in some cases pellets were treated with lysozyme or mutanolysin, in order to weaken the cell wall and facilitate the entry of foreign DNA into the bacterial cell. Cell viability was tested after treatment by growth on plates with these cell wall weakening agents to ensure that they did not kill the bacterial cells. Cells were harvested by centrifugation, resuspended in half the original volume with electroporation buffer and incubated on ice for 10 minutes. Cells were harvested by centrifugation and resuspended in 1/10 the volume with electroporation buffer and incubated on ice for 10 minutes. Cells were harvested by centrifugation and resuspended in 1/100 the volume with electroporation buffer and incubated on ice for 10 minutes. In some instances, cells were treated at 56°C for 5min, 10min, 20min or 30 min. This was done to temporarily inactivate any host restriction systems that would cause degradation of introduced DNA[190, 191]. 100µl aliquots were chilled on ice and used within an hour of preparation.

A1.3.2 Electroporation

Plasmid DNA was isolated from *E. coli* by alkaline lysis method and varying amounts (0.1 ng, 1 ng, 10 ng, 100ng, 1µg, 10µg) of DNA was added to the chilled cells in electroporation buffer. Cells were incubated for 5 minutes with the DNA and then transferred into prechilled electroporation cuvettes (1mm gap or 2mm gap). After incubation, varying pulses were delivered (1000V, 1500V, 2000V, 2500V),

25μF, 200Ω. Cells were immediately recovered in 1mL of iners media and grown at 37°C/5%CO_2 for 2 hours, 6 hours, or 8 hours.

A1.3.3 Modification of plasmid DNA

In some cases, in order to deal with the issue of host restriction modification systems instead of heat inactivating, we attempted an in vitro methylation procedure. Briefly, 500mL of *L. iners* strains (DSM1335, 60B, or 143D) were grown to an OD600 of approximately 1.0 and pelleted by centrifugation. These cells were washed with 100 mL of PENP buffer (10 mM potassium phosphate, 10 mM EDTA, 50 mM NaCl and 0.2M PMSF, pH 7.0). Cells were harvested by centrifugation and resuspended in a final volume of 10mL. The cells were sonicated and debris was pelleted by centrifugation at 4°C. The extract was saved on ice into 1 mL aliquots and 1 mL of glycerol was added as well as BSA (0.2 mg/mL) and stored at -20°C until use. The DNA modification assay was carried out as follows: in a final volume of 100 μl plasmid DNA (100 μg) was mixed with BSA (100 μg/mL), *S*-adenosylmethionine (80 μM), and TNE buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA]. The reactions were incubated at 30°C for 16 hours. These methylated DNAs were then recovered by phenol chloroform extraction followed by ethanol precipitation. Methylated DNA was stored at -20°C until use in the electroporation procedures mentioned above.

A1.3.4 Conjugation

L. iners strains (recipient) were grown either on Columbia blood agar plates or in iners media alone or supplemented with 0.5 M NaCl or 1% glycine for 2 days. Cells were harvested, washed 3 times in iners media. *E.* coli (donor) strains harboring the RK2 helper plasmid with integration vectors were grown in LB overnight and *L. lactis* (donor) harboring pAMβ1 was grown in GM17 overnight and washed in LB or GM17 respectively. In some experiments, the recipient *L. iners* strain was treated with lysozyme or

mutanolysin for 30 min to weaken the cell wall and then washed 3 times in iners media to remove the lysozyme/mutanolysin. After washes, cells were resuspended in 100 µl of the appropriate medium. Recipient (*L. iners*) was spotted onto the plate alone or in combination with the donor strains in varying amounts. Plates were incubated at 37°C/5%CO_2 for 1hr, 2hr, 4hr, 6hr, 8hr, or overnight. Cell mixtures were then retrieved with a swab and plated onto selective media and grown for several days. In some cases, in order to deal with the faster growth rate (and potential overgrowth) of the donor *E. coli* strains, aztreonam and nalidixic acid was included on the plates to prevent their growth and facilitate detection of putative transformants.

A1.3.5 Natural Transformation

After several attempts to introduce plasmid DNA with no success, we attempted to determine if *L. iners* strain DSM13335 could be transformed with genomic DNA. Genomic DNA from the parent strain could possibly be a better transforming factor than plasmid DNA isolated from *E. coli.* We made use of a spontaneous streptomycin resistant mutant of DSM13335. Genomic DNA was isolated from this spontaneous streptomycin mutant using the DNeasy kit (Qiagen). 100mL cultures of *L. iners* were grown for approximately 36 hours (which corresponds to stationary phase), harvested by centrifugation and resuspendend in 1 mL of iners media. Varying amounts of DNA (100 µg, 250 µg, and 500 µg) were added to the bacteria. Cells were incubated with water as a negative control. Cells were incubated at 37°C for varying amounts of time (5min, 30min, 1hr, and 3hr). After the allotted time period, cells were diluted and plated onto Columbia agar +5% sheep blood plates supplemented with streptomycin (40 µg/mL). No differences in the number of streptomycin resistant colony forming units between cells transformed with genomic DNA or water were observed.

A1.4 Conclusions

Ultimately, our attempts to genetically manipulate *L. iners* were unsuccessful. Despite creating numerous vectors for the purpose of knocking out INY, a putative virulence factor for this organism, our results suggest that we were unable to introduce foreign DNA into our recipient *L. iners* strain. Impediments to transformation of Gram-positive organisms include the thick cell wall that can prevent entry of the DNA into the cell and host-restriction modification systems, which can degrade foreign DNA once it gains entry into the cytosol. The genome of *L iners* possesses each of the components of the Type I restriction modification system. We attempted to deal with these issues by treating the cells at 56^{o}C for varying amounts of time to temporarily inactivate host restriction-modification systems[192] before the addition of DNA and electroporation. We also attempted to deal with the host restriction-modification systems by treating plasmids derived from *E. coli* with cell free extracts from the recipient strains, in order to prevent degradation. Additionally, we attempted transformation via conjugation with both a Gram negative (*E. coli*) and a Gram positive (*L. lactis)* donor. Plasmids derived from *E. coli* could potentially be recognized as foreign and be degraded by host restriction modification systems of the organism. We used genomic DNA in order to circumvent this issue, which would have the correct methylation pattern to evade the host restriction system. Transformation of this genomic DNA into the recipient strain should yield more streptomycin resistant transformants than the negative control (as streptomycin resistance could arise as a result of a point mutation during growth). All of our results suggest that *L. iners* strain DSM13335 is recalcitrant to transformation. It is surprising that we were unable to transform this strain as related *Lactobacillus* strains, including *L. crispatus* and *L gasseri* [193, 194] have been successfully transformed. Additionally, an analysis of the genome suggests that this organism has the ability to be transformed as it possess all of the components of the natural transformation machinery, the *com* genes. These genes encode pilus like proteins which serve to bind exogenous DNA, proteins which form channels in the membrane allowing the entry of the DNA, as well

as proteins involved in the creation of ssDNA[195-197]. The inability to transform *L. iners* could be strain specific, and it is worth attempting genetic manipulation with other strains of *L. iners* using the techniques outlined here. Another potential option would be to identify /isolate native plasmids from *L. iners* and utilize these to generate a new set of integration/cloning vectors specific for *L. iners*.

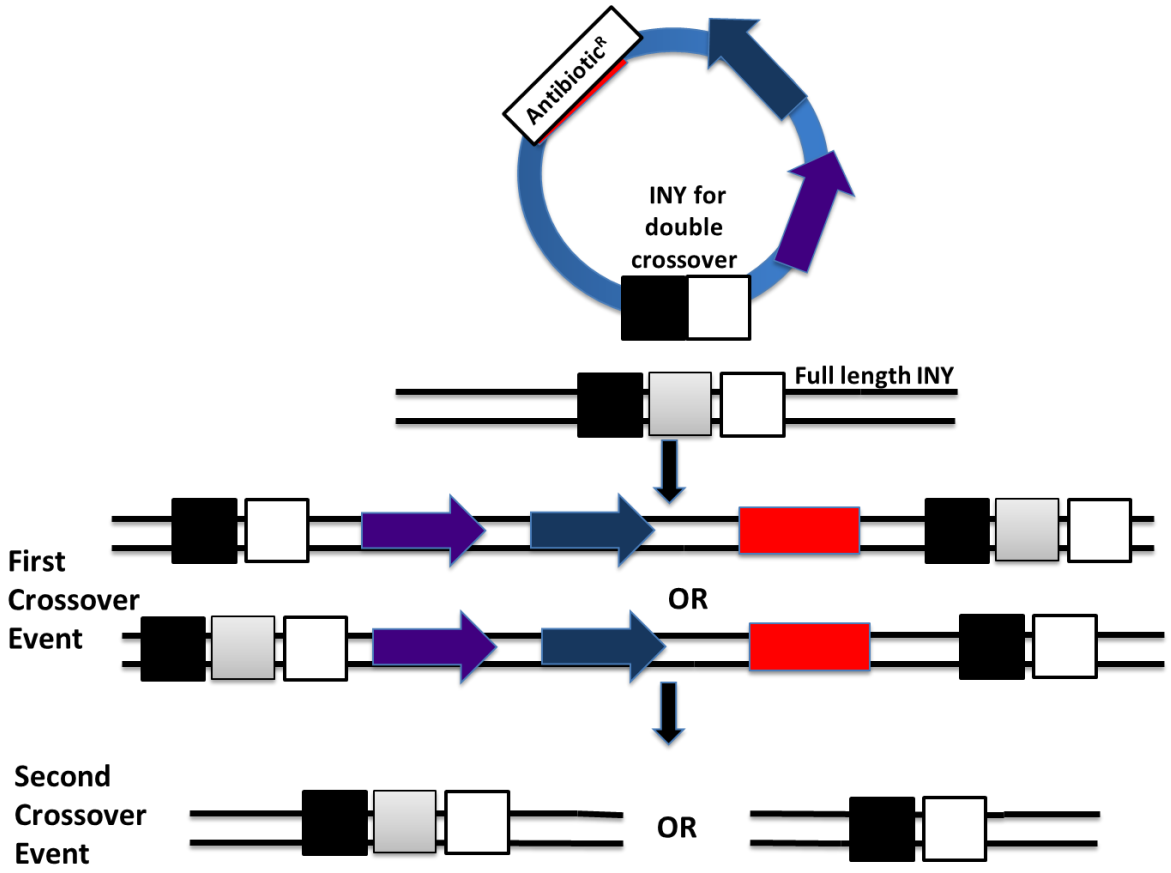

Figure A. Proposed crossover events for integration vectors. Integration vectors containing INY constructs lacking an internal fragment were introduced into the recipient bacteria. In the first crossover event, the plasmid is fully integrated into the genome. This will impart antibiotic resistance. In the second crossover event, the plasmid will loop out, leaving either full length INY or truncated INY in the genome

Appendix B: Heterologous Expression of INY

B.1 Introduction

Our attempts to generate an INY knockout were unsuccessful. In order to address the role of this toxin in contributing to proinflammatory cytokine signaling and defensin production, we sought to utilize a common system for the expression of this putative virulence factor. For over three decades, tools have been developed for the heterologous expression of proteins in *Lactococcus lactis*. This nonpathogenic, noncolonizing microorganism has been extensively used to understand the role of various factors from potentially pathogenic microorganisms in the etiology of disease. This section will outline the attempts made to express inerolysin (INY) in this system, in the hopes of understanding the role of this virulence factor in health and disease of the vaginal tract.

B.2 Materials and Methods

B.2.1 Bacterial Strains and Cell lines

E. coli strains were grown in Luria-Bertani broth. *L. lactis* strains were grown in GM17 broth.

Table B1. Strains and Plasmids used

Strains/Plasmid	Relevant Characteristics	Reference or source
E. coli		
MC1061	araD139, Δ(ara, leu)7697, ΔlacX74, galU-, galK-, hsr-, hsm+, strA	Boca Scientific
TOP10	F mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 deoR recA1 araD139 (ara-leu)7697 galU galK	Invitrogen
TG1	K-12 supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^-m_K^-$)	Stratagene
L. lactis		
MG1363	Plasmid-free and prophage-cured derivative of NCDO 712	[198]
pNZ9000	pepN::nisRnisK; For nisin inducible expression	Boca Scientific
Plasmids		
pKS80	expression vector; constitutive from lactococcal promoter	[188]
pKS80-INY	INY from DSM13335 cloned into BamHI site of pKS80 for constitutive expression	This work
pOri23	expression vector;constitutive expression from p23 lactococcal promoter	Que (2001)
pOri23-INY	INY from DSM13335 cloned into BamHI/SalI sites of pOri23 for constitutive expression	This work
pTRKH3-ermGFP	expression vector; constitutive from erythromycin promoter	Addgene
pTRKH3-ermINY	INY from DSM 13335 cloned downstream of erm promoter replacing GFP for constitutive expression	This work
pNZ8148	nisin inducible expression vector	Boca Scientific
pNZ8148-INY	INY from DSM13335 cloned into KpnI/BamHI sites of pNZ8148 for nisin inducible expression	This work

B.2.2 Cloning and expression of recombinant INY in *L. lactis*

The open reading frame (ORF) encoding INY lacking the predicted signal sequence was cloned from *L. iners* DSM 13335 genomic DNA using primers BamHI-INY-F (GCCGCCGGATCCAATACTGAGCCAAAAACAGCTATTG) and SalI-INY-R (GCCGCCGTCGACTTAGTCATTTTTTACTTCTTCTTTG) for cloning into pOri23. The ORF encoding INY was cloned from *L. iners* DSM13335 genomic DNA using primers SalI-INY-F(GCCGCCGTCGATAATACTGAGCCAAAAACAGCTATTG) and BamHI-INY-R(GCCGCCGGATCCTTAGTCATTTTTTACTTCTTCTTTG) for cloning into pTRKH3-ermGFP. The ORF encoding INY lacking the predicted signal sequence was cloned from *L. iners* DSM 13335 genomic DNA using primer BamHI-INY-F(GCCGCCGGATCCAATACTGAGCCAAAAACAGCTATTG) and BamHI-INY-R(GCCGCCGGATCCTTAGTCATTTTTTACTTCTTCTTTG) for cloning into the BclI site of pKS80. Amplifications were performed using Phusion proofreading polymerase (New England Biolabs). For cloning into pNZ8148, primers KpnI-INY-F (GCCGCCGGTACCAATACTGAGCCAAAAACAGCTATTG) and XbaI-INY-R(GCCGCCTCTAGATTAGTCATTTTTTACTTCTTCTTTG) were used to clone INY. The products of the PCR reaction were cloned via the indicated restriction sites into their respective vectors. For expression in pTRKH3-ermGFP, the GFP was removed by digestion with SalI and BamHI and replaced with INY. Transformation of *E. coli* was carried out as per manufacturer instructions (Boca Scientific). *L. lactis* transformations were carried out as per manufacturer instructions (Boca Scientific).

B.2.3 Nisin inducible expression of INY

L. lactis harboring either pNZ8148-INY (ARLC12) or empty vector pNZ8148 (ARLC13) were grown overnight at 30°C in GM17 with 10µg/mL of chloramphenicol. Overnight cultures were diluted 1/25 into 2x 400mL of fresh medium and grown until OD_{600} reached 0.4 (approximately 4 hours). Cultures were induced with 1 mg/mL of nisin (MP Biologicals) and induced for 2-3 hours. Cells were harvested by centrifugation and pellets were resuspended in PBS and incubated with lysozyme (10 mg/mL) and protease inhibitor for 30 minutes on ice. Cells were sonicated to break them open, and lysate collected by centrifugation. Protein concentrations were determined by modified Bradford assay. Pellets were resuspended in LDS sample buffer (Invitrogen). Recombinant INY was detected by western blot. Samples were also used in an erythrocyte lysis assay.

B.2.4 Detection of recombinant INY from *L. lactis*

For detection of expression of INY from pKS80, pOri23, pTRKH-erm-INY, *L. lactis* was grown in GM17 overnight with 5 µg/mL erythromycin at 30°C. 400mL of overnight culture was harvested by centrifugation, resuspended in 40mL of PBS and treated with lysozyme (1 mg/mL) and protease inhibitor (sigma) for 30 minutes on ice. Cells were sonicated on ice and cells were pelleted by centrifugation. Protein concentrations were determined by a modified Bradford assay (Bio-Rad) and equal amounts of total protein were run on a 4 to 12% polyacrylamide gel (Invitrogen). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% milk and probed with anti-PLY monoclonal antibody (Santa Cruz Biotechnology. 1:1000

dilution). The primary antibody was detected with horseradish peroxidase-conjugated mouse IgG by enhanced chemiluminesence.

B.2.5 Erythrocyte Lysis Assay

The use of human erythrocytes from healthy adult volunteers after verbal informed consent was obtained was approved by the Columbia University Institutional Review Board (protocol IRB-AAAC5641). Defibrinated sheep and horse blood was obtained from Fisher Scientific. Erythrocytes were washed in sterile PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. For endpoint assays, 100 µl of a 1% washed erythrocyte solution was mixed with 100 µl of lactococcus lysates or supernatant in a 96-well V bottom plate and incubated for 30 min at 37°C and 5% CO_2. After 30 min, the plates were spun at 2,000 rpm to pellet erythrocytes. Supernatant was removed, and the optical density at 415 nm (OD415) was measured.

Figure B1

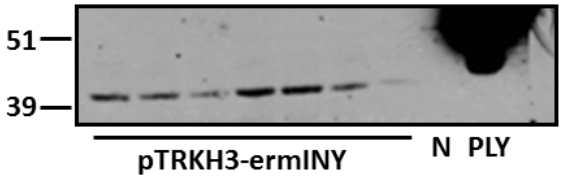

Figure B2

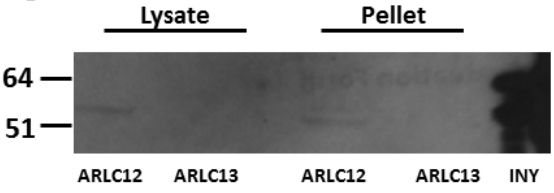

Figure B3

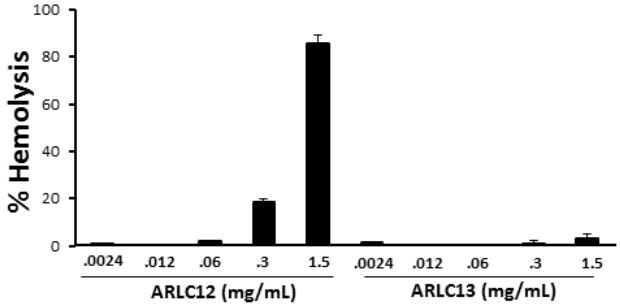

Figure B. **Production of rINY by *Lactococcus lactis*. (B1)** Expression of INY from pTRKH3ermINY was assessed by western blot using an anti-PLY antibody. **(B2)** Expression of INY from ARLC 12(*L. lactis* containing pNZ8148-INY) and ARLC12 (*L. lactis* containing empty vector pNZ8148) was assesed by western blot using anti-PLY antibody. **(B3)** Lysates from ARLC12 and ARLC13 were used in an erythrocyte lysis assay.

B.3 Results

Interestingly, despite cloning the ORF encoding INY into pKS80 and pOri23, which should result in constitutive expression, we were unable to detect INY in lysates or pellets of these transformants. Analysis of *L. lactis* strains harboring the plasmid pTRKH3-ermINY by western blot revealed the presence of an approximately 39kDa protein which cross-reacted with an anti-PLY antibody (Figure B1). Although cross reactive, this protein was not consistent with the predicted size of INY (57kDa). These lysates were found to have no lytic activity despite the addition of milligrams of total protein (data not shown). In contrast, lysates from *L. lactis* harboring the pNZ8148-INY (ARLC12) contained an approximately 57kDa protein which was cross reactive with an anti-PLY antibody, consistent with the expression of INY from this strain.

This was unique to *L. lactis* harboring the construct, as cells harboring the empty vector (ARLC13) did not produce any protein recognized by the anti-PLY antibody (Figure B2). In a functional erythrocyte lysis assay, we confirmed the presence of functional pore forming toxin in ARLC12 lysates as they were able to induce hemolysis of red blood cells (Figure B3). Conversely, ARLC13 lysates, which possessed no toxin were not able to induce lysis when comparable amounts of total protein were used. We attempted to use these strains to induce production of proinflammatory cytokines from VK2 cells. However, we were unable to detect any significant differences between strains (data not shown). We hypothesize that intracellular components of the bacteria were able to induce production of proinflammatory cytokines, given we used lysates of the bacteria. Thus these constructs proved to be less useful than expected in addressing the role of this toxin in these epithelial cell responses.

B.4 Discussion

Lactococcus lactis is commonly used as a tool for studying the role of putative virulence factors in disease pathogenesis. Our goal was to express inerolysin in a related lactic acid bacteria (LAB) to understand the role of this toxin in proinflammatory cytokine production and to shed light on the role of this putative virulence factor in the pathogenesis of bacterial vaginosis (BV) and BV associated preterm labor. We were unable to detect expression in strains harboring the constitutive expression vectors pKS80 and pOri23. With the use of the constitutive expression vector using the erythromycin promoter, we obtained protein which cross reacted with antibody raised to a related CDC, pneumolysin. This cross-reactive protein however was not the expected size of INY (approximately 57kDa). When tested in a hemolysis assay, these

proteins were not able to induce any significant lysis, indicating production of a truncated protein. We hypothesize that constitutive expression may put undue stress on the cell, providing pressure for recombination of the introduced plasmid, resulting in a truncated non-functional protein. Alternatively, constitutive expression may lead to intracellular accumulation of toxin, which may then be subject to degradation by intracellular proteases. This is often a problem, which led to the development of inducible vectors for expression, allowing for growth to high density followed by shorter periods of induction to prevent excessive intracellular accumulation[199]. Using the nisin inducible expression vector pNZ8148 we were able to express full length, functional INY in this LAB. However, when we used these constructs to stimulate vaginal epithelial cells to address the role of INY in initiating proinflammatory cytokine signaling, we found that our empty vector control also induced the same level of signaling. It is not clear why this was the case, but it is possible that some soluble factor present in the cytosol of *L. lactis* has proinflammatory effects. To address this possibility, we attempted to create INY fused to the usp45 secretion signal. Usp45 is the major secreted protein from *L. lactis* and this strategy has been widely used to target proteins for secretion. Unfortunately, our attempts to create this construct were unsuccessful. Overall, while we were able to achieve heterologous expression of the toxin in a related LAB, this strategy proved to be of little use for studying the epithelial cell responses to INY.

Appendix C: Additional Figures for pH dependent Activity

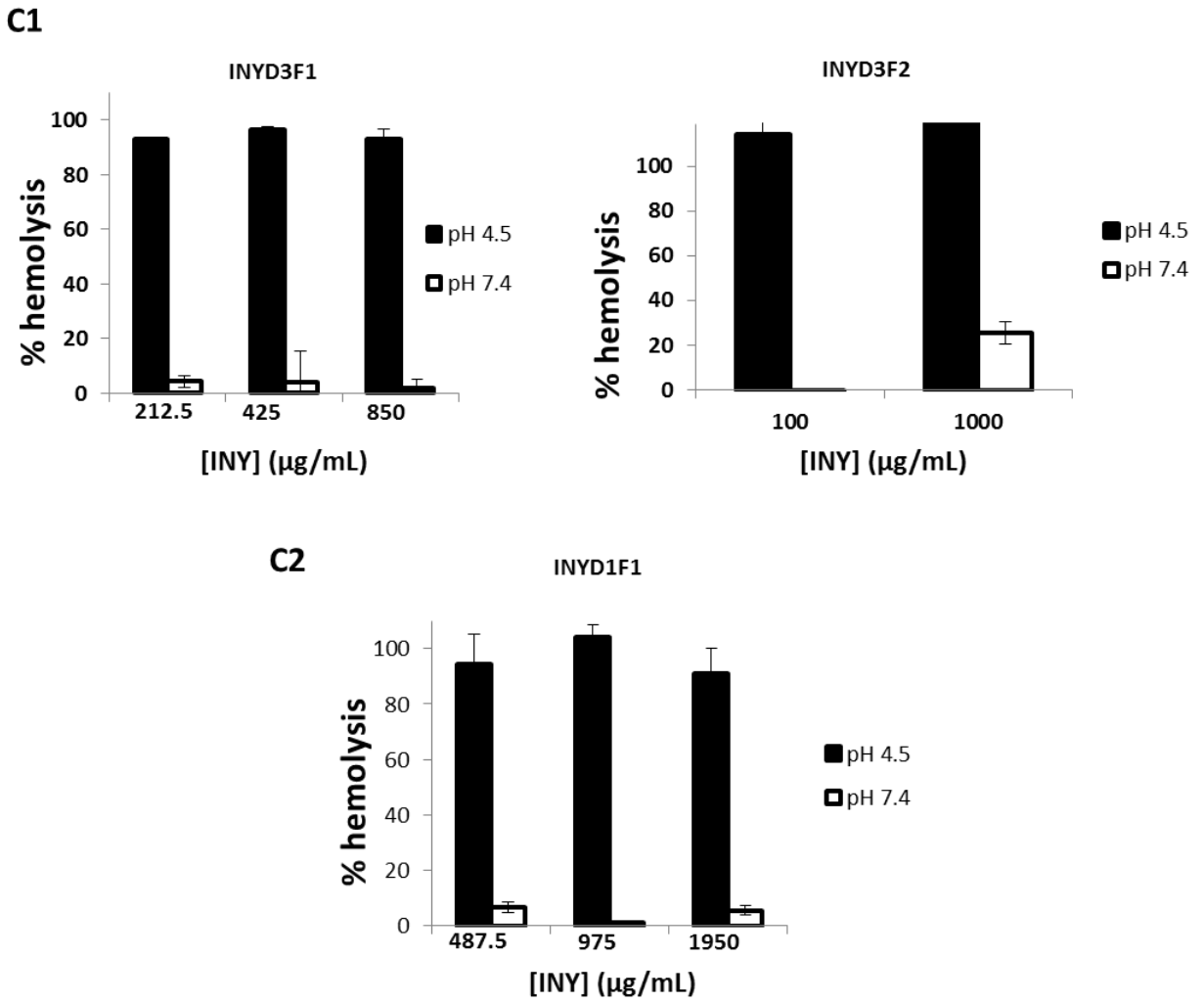

Figure C. Additional Figures for pH dependent activity. Toxins which displayed the "aggregation" phenotype were used. **(C1)** INYD3F1 INYD3F2 and **(C2)** INYD1F1 were preincubated for 30 min at 37°C at the indicated pH before use in a hemolysis assay