CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

Heterologous Expression and Secretion of *Aggregatibacter* Leukotoxin A by a Tumor-Targeted *Salmonella*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology

by

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August 2017

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ACKNOWLEDGEMENTS

I would like to thank Scott Kachlany for generously donating the anti-LtxA antibody used in this work, my mentor David Bermudes for welcoming me into his lab and supporting me throughout my studies at CSUN, David Quintero for his help in troubleshooting key experiments, Jamie Carrafa and Ranelle Buck for their company in lab, my committee members Rheem Medh and Michael Summers for their guidance, the CSUN MARC/RISE program and the CSUN office of graduate studies for their financial support over the course of my graduate degree, and my family.

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ABSTRACT

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Despite the advancement of numerous treatment options, conventional cancer therapeutics generally fail to prevent tumor recurrence or successfully treat metastatic cells. A promising alternative involves the use of attenuated *Salmonella* strains, which preferentially replicate within tumor tissue and suppress tumor growth. Although the tumor-targeted strain VNP20009 localized to some tumors with high efficiency in human clinical trials, they failed to result in eradicating cancer cells or delay tumor growth. To enhance the tumoricidal efficacy of this strain, we engineered the bacteria to express leukotoxin A (LtxA), a pore-forming multigene toxin produced by *Aggregatibacter actinomycetemcomitans* with natural specificity for β_2-integrins found on human leukocytes. Previous studies have shown that the overexpression of this surface antigen on cancer cells is associated with increased metastatic potential, suggesting that LtxA may represent a target for metastatic tumors, which are notoriously resistant to therapy. The LtxA-encoding genes were cloned into the inducible plasmid vector pAra99a and expressed in the tumor-targeted *Salmonella* strain VNP20009. LtxA expression at the

mRNA transcript level was verified by RT-PCR. A codon-optimized LtxA sequence was generated and tested for its ability to enhance expression in the VNP20009 background. To improve the secretion of the LtxA protein into the culture supernatant, the wild-type LtxCA was co-expressed with the *E. coli* hemolysin-α (HlyA) secretion complex, HlyBD. C-terminal modifications were generated to facilitate interaction of the LtxA protein with the HlyBD complex. Protein expression of the wildtype and modified LtxA constructs generated in this study could not be consistently determined by immunoblot analyses, although Columbia agar hemolysis assays suggested that active LtxA protein was functionally expressed and secreted by the wild type LtxCABD construct and an LtxCA with a modified C-terminus together with the *E. coli* HlyBD expressed in trans. In preliminary experiments, the human monocytic cell line THP-1 was sensitive to wildtype LtxCABD and to the C-terminal fusion constructs when co-expressed with HlyBD by VNP20009.

INTRODUCTION

Cancer prevalence and the limitations of conventional treatments

Cancer remains one of the leading causes of death in the developed world. In the United States, it is second only to heart disease in total deaths per year and is the leading killer of adults between the ages of 40 and 79 (Siegall et al., 2016). Although many advances have been made in the characterization of cancer progression and the development of therapeutic options, radiation and chemotherapy fail in eradicating entire cancer cell populations in a patient and cause side-effects to normal healthy tissues. These limitations are largely due to variation in tumor characteristics among patients, between tumor types, and within tumor cell populations themselves (Kiberstis and Travis, 2017). Other shortcomings, such as the lack of early detection methods, procedure invasiveness, and high patient costs underscore the need for alternative solutions, and efforts continue to be made in numerous fields to develop them (St. Jean et al., 2008).

Alternative treatment methods must demonstrate (1) high tumoricidal efficacy, and (2) target specificity. A promising approach to address these requirements lies in *how* an anti-cancer drug is delivered to the tumor site. Most chemotherapeutic drugs available to patients today only kill actively dividing cells that tend to accumulate at periphery of a tumor. As a result, quiescent cells located in the inner regions of the tumor characterized by lack of vascular elements are not affected (Kasinskas et al, 2006). In addition to increased tumor-targeting, controlled delivery can enhance the efficacy of a drug by overcoming the interstitial pressure that otherwise limits access to the tumor (Azzi et al., 2013). The inability to reach these distant sites and selective ablation of accessible phenotypes can result in a drug-resistant phenotype through the selection of cell variants

with enhanced resistance to drug-induced apoptosis. Mechanisms of drug resistance include cell permeability alterations, overexpression of drug efflux pumps, aberrant DNA-damage repair pathways, and differential expression of target receptors (Gottesman, 2002). These complications highlight the need for alternative treatments based on effective drug-delivery systems that direct cancer compounds directly to the tumor.

Bacterial Therapeutics

A promising approach involves the use of bacteria as tumor-targeted delivery vector. The notion of using bacteria in this manner can be traced back to the late 1800's when Vautier observed tumor shrinkage in patients also suffering from infection with the pathogenic bacterium *Clostridium perfringes,* the causative agent of gas gangrene (Wei et al, 2008). Wilhelm Busch later noted tumor regression in patients with erysipelas, a skin condition resulting from *Streptococcus pyogenes* infection (Patyar et al, 2010). This prompted Friedrich Fehlesien, and later, William Coley to deliberately infect patients with inoperable tumors with live *S. pyogenes,* and his experiments revealed a correlation between fever and tumor regression. At the time, it was not understood how exactly the bacterial infections caused tumor regression, but it is now believed that the bacteria triggered an immune response leading to tumor shrinkage.

Coley subsequently altered his approach by using bacterial extracts derived from *S. pyogenes* and *Serratia marcescans*, known as Coley's toxin, instead of live bacteria which sometimes caused uncontrollable infections. The outcome of studies using Coley's toxins included the complete regression of different types of solid tumors (Cann et al., 2003). These findings were ultimately responsible for initiating a class of therapeutics

known as immunotherapy. Later, the ability of bacteria to selectively replicate in solid tumors led to the use of live bacteria to treat cancer and is the basis for clinical trials using different engineered bacterial strains including *Clostridium* (ClinicalTrials.gov Identifier NCT01118819) and *Salmonella* (ClinicalTrials.gov IdentifierNCT01099631).

***Salmonella typhimurium* strain VNP20009**

Salmonella, as facultative anaerobes, offer several advantages over anaerobic delivery vectors such as *Clostridium*. The bacteria's ability to tolerate oxygen enables them to invade secondary metastatic tumor sites, yet they can grow in anoxic environments, allowing them to colonize low oxygen environments present in tumor due to improper circulation (Forbes, 2010). Once in the tumor, *Salmonella* are shielded from the host immune response due to poor tumor vascularization, which contributes to their preferential accumulation within the tumor (Forbes et al, 2003). In addition, the bacteria secrete a biofilm layer within necrotic tissues of the tumor, which acts as a protective barrier against immune cells (Crull, et al., 2011). *Salmonella* expression of serine, aspartate and ribose/galactose receptors and the availability of their ligands at the tumor site appear to be involved in tumor tropism, while the expression of specific surface glycans by cancer cells are known to attract the bacteria (Kasinskas and Forbes, 2007 and Wang et al., 2016).

Tumor-specific bacteria are derived from pathogenic bacteria and must be stripped of their virulence factors while maintaining or enhancing their tumor-invading capacity in a process known as "attenuation". To date, several groups have developed tumor-targeted strains of *Salmonella* (Hoffman, 2015). However, the use of these strains in the clinic is limited by several serious safety concerns including systemic toxicity, off-

target effects, and antibiotic resistance. To address these issues, the attenuated strain of *Salmonella typhimurium* VNP20009 was constructed through the deletion the *msbB* gene, which inhibits Lipid-A mediated septic shock in the host (Low et al., 2004), the *purI* gene, which ablates prototrophic purine production rendering the bacteria unable to grow in the absence of an exogenous adenine source, and the Suwwan deletion, a spontaneous *msbB* suppressor mutation, to genetically stabilize *msbB*$^-$ mutants (Murray et al., 2004). VNP20009 has been shown to target solid tumors and cause tumor growth inhibition in mice using murine and human tumor cell xenografts (Luo et al, 2001).

In a Phase I clinical trial in which metastatic melanoma patients were administered VNP20009 intravenously, the bacteria demonstrated the ability to safely target solid tumors in some patients, but did not contribute to tumor growth retardation as seen in previously in murine models (Toso et al., 2002). In an attempt to increase tumor toxicity, VNP20009 research has moved in the direction of optimizing the bacteria as drug-delivery vectors rather than relying on their intrinsic tumoricidal properties. VNP20009 possess many attributes sought after in drug-delivery vectors, including motility, the ability to replicate within solid tumors, to remain metabolically active, and to be readily removed after treatment using antibiotics (Forbes, 2010). Examples of cargo used in the VNP20009 background include enzymes which convert pro-drugs to an active form at the tumor site (Pawelek et al., 1997; King et al., 2002), apoptosis-inducing signaling molecules (Jeong et al. 2014; Ganai et al., 2009), and therapeutic proteins expressed and secreted into the extracellular space (Zheng et al., 2000; Van Dessel et al., 2015; Quintero et al., 2016).

Heterologous expression of bacterial toxins in VNP20009

Due to their genetic tractability and natural ability to kill tumor cells, several bacterial toxins have been expressed in tumor-specific *Salmonella* strains. Examples include the α-hemolysin of *Staphylococcus aureus* (SAH; Swofford et al., 2014) and *Escherichia* /*Salmonella* cytolysin A (ClyA; Jiang et al., 2013). However, these toxins have the potential to cause systemic toxicity because of their lack of specificity, even when expression and delivery are controlled using tumor-targeted vectors. Our laboratory has recently developed EGFR-specific chimeras of *Pseudomonas aeruginosa* Exotoxin A (ToxA) and successfully shown that powerful toxins like ToxA can be modified to target EGFR-positive human cancer cells in a VNP20009 background to lower the risk of systemic toxicity while retaining their ability to efficiently kill cells (Quintero et al., 2016). To date, several bacterial toxins with natural specificity toward surface receptors overexpressed in cancer cells have been characterized, but none have been extensively tested in a tumor-targeted *Salmonella* background. Thus, inherently tumor-specific bacterial toxins remain to be explored for their applications to bacterial- based cancer therapies.

***Aggregatibacter actinomycetemcomitans* leukotoxin A (LtxA)**

An example of a toxin with a naturally occurring tumor specificity is the *Aggregatibacter actinomycetemcomitans* leukotoxin A (LtxA). Kachlany et al. have purified the toxin as a therapeutic agent known as Leukothera™, which has been successful in treating hematological diseases, including leukemia and lymphoma, in animal models (Kachlany et al., 2010; DiFranco et al., 2015). The natural target for LtxA is the beta-chain subunit of β_2-integrin adhesion molecules found on human leukocytes

and macrophages (Dileepan et al., 2007). LtxA is effective against lymphoblastic and myeloid cancers due to the overexpression of β_2-integrins by representative cell lines, making it one of few bacterial toxins characterized thus far with inherent specificity to surface markers overexpressed in cancer. LtxA may also be able to target highly invasive variants of solid-tumors, as it has been shown that β_2-integrin expression is associated with increased metastatic potential in murine models (Larizza et al., 1984). Table 1 summarizes the various types of malignancies that LtxA may be able to target, including hematological cell lineages with known sensitivity to the toxin. Solid tumor lines with β_2-integrin expression that have not been tested for sensitivity to LtxA are highlighted.

A. actinomycetemcomitans is a gram-negative bacterium implicated in several human diseases including localized juvenile periodontitis (Slots et al. 1980). One of the virulence factors of this organism is LtxA, a member of the repeat-in-toxin (RTX) family of CA^{2+}-dependent cytolytic toxins produced by a large group of gram-negative pathogens, including the *E. coli* hemolysin (HlyA) and *M. haemolytica* leukotoxin (LktA; Coote, 1992). RTX toxins are characterized by a common CABD operon structure and the presence of glycine-rich nonapeptide repeats near the C-terminus of the structural protein, which vary in number depending on the toxin and are required for cytotoxicity. There is a wide range of species and cell-type specificity within the RTX toxins, some being toxic to a relatively large number of cell types in various species such as the *Actinobacillus pleuropneumoniae* ApxI hemolysin, which is toxic to porcine erythrocytes (Frey, 1995), while others only affect specific cell types in several

Cell line	Type	β_2-integrin expression	LtxA-sensitive	Reference
HL-60	promyelocytic leukemia	++	Y	Simpson et al., 1988
THP-1	monocytic leukemia	+++	Y	Simpson et al., 1988
K-562	erythroleukemia	-	N	Simpson et al., 1988
JURKAT	T cell leukemia	++	Y	Simpson et al., 1988
Daudi	B cell lymphoma	+	Y	Simpson et al., 1988
U-937	monocytic lymphoma	+++	Y	Simpson et al., 1988
MDA-MB-231	breast adenocarcinoma	++	N/A	Kawai et al., 2009
MCF-7	breast adenocarcinoma	+	N/A	Kawai et al., 2009
EFO-21	ovarian cystadenocarcinoma	+++	N/A	Human Protein Atlas
RH-30	rhabdomyosarcoma	++	N/A	Human Protein Atlas
A-549	lung carcinoma	+	N/A	Human Protein Atlas

Table 1. β_2-integrin expression and LtxA-sensitivity in hematopoietic cancer and solid-tumor cells.

organisms such as *M. haemolytica* LktA, which only lyses ruminant leukocytes (Wilkie & Shewen, 1988). LtxA is unique among this group in that it is specific only to human and new-world primate leukocytes and phagocytes (Taichman et al., 1987), although it can also affect erythrocytes and lymphocytes at high toxin concentrations (Mangan et al., 1991) or when using a specific type of media such as Columbia agar with sheep red blood cells (Balashova et al., 2006).

The *LtxA* operon consists of the leukotoxin structural gene, known as the "*A*" gene, two transport genes "*B*" and "*D*", and a post-translational acylating gene "*C*" (Lally

et al., 1989; Kraig et al., 1990; Figure 1). The 116 kDA LtxA protein can be further divided into 4 regions (Figure 2); the N-terminal domain, which spans the first 408 residues and interacts with the host bilayer to form a pore, the central region which contains lysines 562 and 678 that require acylation by LtxC to activate LtxA (Balashova et al., 2009), the 14 glycine-rich nonapeptide repeats region required for proper interaction with the LtxAtarget receptor (Lally et al., 1994), and the C-terminal region necessary for interaction with LtxB and LtxD and export through the periplasmic space (Lally et al., 1991) (Figure 2). *LtxB* encodes an ATP-binding cassette-containing inner membrane protein that interacts directly with the LtxA C-terminus. LtxD is a transmembrane protein and allows passage of LtxA into the periplasmic space, as well as recruits TolC-homolog TdeA which forms a trimeric pore in the outer membrane protein via the LtxBD/TdeA complex. (Crosby & Kachlany, 2007) (Figure 3).

The known receptor for LtxA is human CD18 (ITGB2), the beta-chain subunit of β_2-integrin adhesion molecules found on human leukocytes and macrophages (Dileepan et al., 2007). These include lymphocyte-function associated antigen (LFA-1) and macrophage-1 antigen (Mac-1). At high doses, LtxA interacts with the host plasma membrane via β_2-integrins by anchoring itself to the bilayer by two acyl groups found in its central region and forms pores, triggering lysis through membrane collapse and rapid necrosis (Korostoff et al., 1998; Figure 4). Lower doses of LtxA trigger apoptosis in human leukocytes and monocytes (Tsai et al., 1979). Very low concentrations of LtxA were shown to induce lysis via monocyte secretion of interkeukin-1β (IL-1β) and activation of capsase-1 (Taichman et al., 1980). In Jurkat T cells, LFA-1 units cluster into lipid rafts, triggering a signal transduction pathway that results in mitochondrial release of cytochrome c and activation of caspase-7 and -9 (Fong et al., 2006). In other cells,

such as the promyelocytic leukemia line HL-60, LtxA causes cell death by a reduction in mitochondrial potential and reactive oxygen species production, which activate caspase-dependent apoptosis (Korostoff et al., 2000).

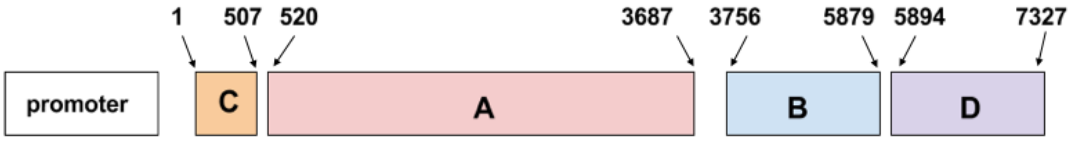

Figure 1. **Organization of the *Aggregatibacter* LtxA operon.** The LtxA toxin is flanked by the genes encoding the LtxC acylation protein and the LtxB and LtxD secretion complex. Numbers indicate nucleotide position of coding regions.

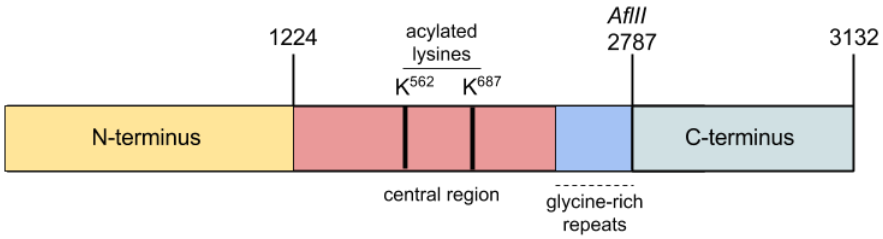

Figure 2. **LtxA structural protein domains**. The LtxA protein can be divided into the N-terminus, the central region, including acylated K^{562} and K^{687}, the repeat region which ends at the *Afl*II (nucleotide 2787) restriction site, and the C-terminus ending a nucleotide 3132.

Heterologous expression of LtxA in VNP20009

In this work, the antitumor potential for *A. actinomycetemcomitans* LtxA was further exploited by engineering the tumor-targeted strain of *Salmonella typhimurium* VNP20009 to produce the toxin. A *Salmonella* capable of expressing and secreting LtxA as an oncolytic agent could address the limited efficacy of the attenuated strain by enhancing the anti-tumor efficacy *in vivo* while maintaining its established safety profile. It could also represent a potential means for targeting metastatic cells, which are notoriously resistant to conventional cancer therapies.

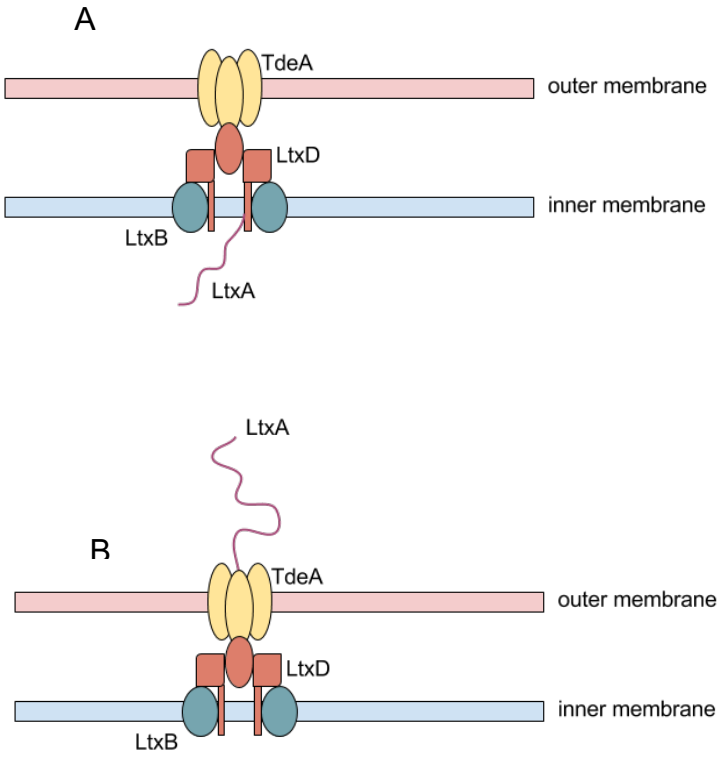

Figure 3. Secretion of the LtxA structural toxin via the LtxBD/TdeA complex. (A) Binding of the LtxA C-terminus to the LtxB ATP binding cassette. (B) Secretion of LtxA through the outer membrane TolC homologue TdeA.

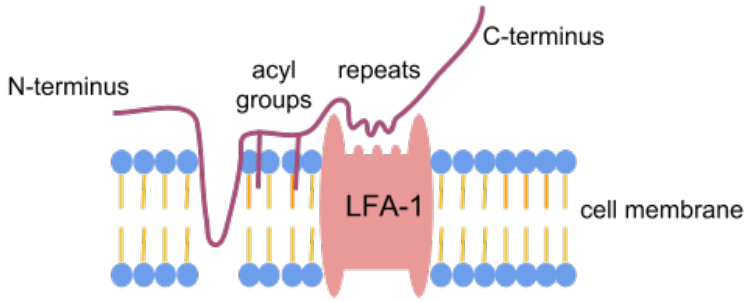

Figure 4. **Interaction between LtxA and target receptor LFA-1.** The repeat region binds specifically with the LFA-1, while the acyl groups become anchored into the plasma membrane and the pore-forming portion of the protein inserts through the bilayer.

MATERIALS & METHODS

Bacterial strains and plasmids

Lyophilized *A. actinomycetemcomitans* strain CDC B5386 [NCTC 10982] (ATCC #29524) was used as a DNA template for LtxCA and LtxCABD PCR amplification and cloning. HK1651 [RAP-1] (ATCC #700685), a highly leukotoxic JP2 clone of *A. actinomycetemcomitans,* which harbors a 530bp promoter deletion thought to remove a repressor binding site and lead to increased expression of LtxA (Haubek, 2010), was used as positive control for LtxA secretion. B5386 and JP2 were grown on chocolate agar (Hardy Diagnostics) at 37°C in a 5% CO_2 atmosphere. For liquid cultures, *A. actinomycetemcomitans* General Media (AAGM; 4 % w/v tryptone, 0.6% w/v yeast extract, 0.8% w/v dextrose; Fine el al., 1999) was buffered with 0.4% w/v sodium bicarbonate ($NaHCO_3$) immediately prior to inoculation for optimal LtxA-secretion (Kachlany et al. 2002). All strains were stored in 15% glycerol at -80°C.

The *Escherichia coli* EC100 cloning strain was used for amplification and purification of LtxA-containing plasmids prior to transformation into *Salmonella enterica* serotype *Typhimurium* restriction (-) methylation (+) strain (Tsai et al., 1989) and attenuated *Salmonella enterica* serotype *Typhimurium* strain VNP20009 from the American Type Culture Collection (#202165). The *E. coli* Rosetta DE3 derived from BL21 was assessed for its ability to enhance heterologous protein expression of LtxA. *E. coli* and *Salmonella* strains were grown at 37°C in Luria-Bertani (LB; 1% tryptone, 0.5% yeast extract, and 1% NaCl +1.5% agar for plates; Miller, 1992), or LB lacking salt (LB-0; Murray et al., 2001) supplemented with 100 μg/mL ampicillin (Amp_{100}) and/or 30 μg/mL chloramphenicol (Cam_{30}) depending on the bacterial strained used.

Organism	Strain	Genotype	Source/Reference
Escherichia coli	EC100	*F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ – rpsL (StrR) nupG*	Epicentre, Madison, WI
Escherichia coli	Rosetta DE3 pLysS	*F- ompT hsdSB(rB- mB-) gal dcm (DE3) pRARE CamR*	Michael Summers (CSUN)
Salmonella enterica sv. *Typhimurium*	JR501	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 H1-b H2-e,n,x (cured of Fels 2) fla-66 nml rpsL120 xyl-404 galE719*	*Salmonella* Genetic Stock Center, Calgary, CA/Tsai et al., 1989
Salmonella enterica sv. *Typhimurium*	VNP20 009	*msbB purI*; Suwwan suppressor deletion	ATCC 202165
Aggregatibacter actinomycetemcomitans	B5386		ATCC 29524
Aggregatibacter actinomycetemcomitans	JP2	530bp LtxA promoter deletion; *tad* mutant	ATCC 700685

Table 2: bacterial strains used in this study

The expression plasmid pAra99a used in these studies was derived from pTrc99a (Amann et al., 1988; GenBank U13872.1) where the *trc* promoter and lacIq were replaced with the arabinose-inducible operon AraBAD, which was obtained from pBAD- IEE (GenBank AB598835; Quintero et al., 2016). The chloramphenicol-resistance pLG575 plasmid carrying the *E. coli* HlyBD genes was derived from pACYC184 by insertion of the HlyB and HlyD coding sequences into the *tet* gene (Mackman et al., 1989).

Preparation of electrocompetent bacteria

Electrocompetent JR501 and VNP20009 cells were prepared according to a modified protocol modified based on Enderle and Farwell (1998) using LB-0, which improves transformation efficiency (David Quintero, personal communication). Freshly streaked bacteria were incubated overnight at 37°C on LB, re-streaked the following day on LB-0, and allowed to incubate overnight at room temperature. 1 mL of sterile ice-cold sterile 1% glycerol was added directly to the plate and the cells loosened with sterile a loop, followed by harvesting of the cell suspension by centrifugation. The pellet was washed 5 times with ice-cold 1% glycerol and re-suspended in 10% glycerol at 10 times the pellet volume, aliquoted and stored at -80°C.

PCR and cloning

PCR reactions were performed using the high-fidelity polymerase KOD Hot Start Mastermix (Novagen/EMD Millipore, Billerica, MA) using the primers listed in Table 3 (Integrated DNA Technologies, San Diego, CA). Generally, programs consisted of one cycle of 95°C for 2 min followed by 35 cycles of 95°C for 10s, 58°C for 20s and 72°C for 15– 30 s/Kb, with a final extension of 72°C for 5 min. The resulting PCR products were resolved on 0.7%-1.5% agarose gels run in Tris-acetate ethylenediaminetetraacetic acid EDTA (TAE) buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) with 1:10,000 of a 1% solution of ethidium bromide or 10X SYBR-Safe (Invitrogen) added to the gel for visualization. PCR bands were gel purified using Ultrafree MC 0.45 μM PVDF membrane cartridges (Merck Millipore, Cork, Ire.), precipitated with sodium acetate and ethanol, and reconstituted in sterile nanopure water. The LtxA sequence was amplified using lyophilized B5386 cells (ATCC 29524)

as the DNA template. Following amplification, the LtxA fragment was digested with the appropriate restriction endonucleases (Fermentas) as listed in Table 3, and ligated into the pAra99a vector using T4 DNA ligase (Fermentas). The constructs were transformed into chemically competent EC100 cells and grown on LB Amp_{100} plates overnight following a 1h recovery period in SOC media (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). Plasmids were isolated from clones (GeneJET Plasmid Miniprep Kit, Fermentas) and screened by restriction analysis prior to Sanger sequencing of both strands (Sequetech, Mountain View, CA).

The validated constructs were electroporated into *Salmonella* JR501 strain using a BTX Electro Cell Manipulator 600 (1.70 kV charging voltage; 2.5 kV resistance; 25 μF capacitance timing; 129 Ω resistance timing) to establish the proper methylation pattern required for expression in VNP20009 (Tsai et al., 1989). The LtxA-containing plasmids were purified from *Salmonella* JR501 lysates (Promega) treated with an alkaline protease solution and transformed by electroporation into VNP20009 using the same conditions listed above.

Overlapping PCR

Overlapping PCR's (Hoover and Lubkowski, 2001 and Stemmer et al, 1995) were performed using an initial PCR reaction with the KOD Mastermix (Novagen/EMD Millipore, Billerica, MA) for the LtxA and hemolysin-α (HlyA) sequences from the strains listed in Table 2, the products of which were gel purified as described in the previous section. Approximate equimolar concentrations of each reaction were mixed into the first round overlapping PCR for a total of 15 cycles. 1 µL of the first round

PCR was then introduced into a second round of PCR (50 µL total reaction volumes, 30 cycles) with the outer most primers. Overlapping PCR products were then purified by gel electrophoresis, digested with the appropriate restriction enzymes, cloned into pAra99a-LtxCA using *Afl*II and *Sma*I, and verified by Sanger sequencing.

Protein precipitation

Proteins were precipitated using equal volumes of pyrogallol red molybdate methanol (PRMM; 0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50.0 mM succinic acid, 20% vol/vol methanol; pH=2.0 (Caldwell and Lattemann, 2004), incubated for 2 hours at room temperature followed by 4°C overnight, and centrifuged at 10,000 x g for 1 hour at 4°C. Pellets were rinsed with acetone twice and allowed to dry, resuspended in sodium dodecyl sulfate (SDS) reducing buffer (0.5 M Tris-HCl pH=6.8, 26% vol/vol glycerol, 10% w/vol SDS, 0.5 w/vol bromophenol blue) supplemented with 160 mM dithiothreitol (DTT), and boiled for 5 min at 98°C.

Preparation of culture supernatants

For *Salmonella* supernatants, JR501 and VNP20009 were grown in (0.2% casamino acids, 0.2% tryptone, 0.1% yeast, and 0.9% NaCl; buffered to pH=7.4 with 0.2M NaOH) supplemented with 50 µg/mL ampicillin, which was formulated to minimize toxicity against mammalian cells (Quintero et al., 2016). The cultures were grown at 37°C for with 15RPM rotation for 8-10h, diluted 1:5 in fresh CTY and incubated for a 2-hour pre-induction period prior to being induced with 1 µg/mL of filter- sterilized L-arabinose at 37°C with rotation for 4 hours. The cultures were

centrifuged for 15 min at 3000 x g at room temperature. The supernatants were harvested and sterilized using a 0.2 μM low-binding PES filter (Whatman, Buckinghamshire, UK; Puradisc 25 AS). Supernatant proteins were PRMM precipitated as outlined in the previous section.

Isolation of periplasmic periplasmic proteins

Periplasmic proteins were isolated by pelleting cells at 10,000 x g for 10 min at 4°C, resuspending in 1:50 volume Tris-sucrose EDTA buffer (TSE; 200 mM Tris-HCl pH=8, 500 mM sucrose, 1 mM EDTA; Delcour, 2013), incubating on ice for 30 min, and spinning at 10,000 x g for 30 min at 4°C to harvest the resulting supernatant. Periplasmic proteins were precipitated using PRMM as described above.

Preparation of cytoplasmic fractions

Cells were grown in LB broth with 50 μg/mL ampicillin overnight at 37°C for with 15 RPM rotation. The cultures were diluted 1:5 in fresh LB media and grown for 2 hours before being induced with filter-sterilized 1μg/mL L-arabinose at 37°C with rotation for 4 hours. Cell pellets were harvested and the supernatants removed prior to resuspension in 10 times the pellet volume of sample reducing buffer with 160 mM DTT. The suspensions were then passed through a 28G needle (Becton-Dickinson) 4-5 times and boiled at 98°C for 5 min.

Immunoblots

Protein samples prepared from cell pellets, periplasmic regions, and supernatant fractions were resolved on 10% Tris-glycine polyacrylamide gels run at 100 to 120 volts. Proteins were then transferred to 0.45 μm PVDF membrane (Immobilon-FL,

Millipore Corp.) overnight at 30 volts at 4°C and blocked with 5% non-fat milk in T-TBS washing buffer (0.05% Tween 20, 50 mM Tris Base, 0.5 M NaCl, pH 7.6) for 45 minutes, followed by an incubation at room temperature for 3 hours with a mouse anti-LtxA monoclonal antibody (donated by Scott Kachlany, Rutgers University) diluted 1:10,000 in 3% bovine serum albumin (BSA) in TBS-T. The primary antibody was washed three times prior to incubating with a rabbit anti-mouse horseradish peroxidase-conjugated antibody (Jackson Laboratories, West Grove, PA) diluted 1:20,000 in blocking buffer for 1 hour at room temperature. The wash step was repeated and the protein detected by enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescent Substrate, Thermo Scientific).

RNA isolation and RT-PCR

Cells induced with L-arabinose for 4 to 16 hours were harvested by centrifugation at 3,000 x g for 10 minutes and homogenized in 750 µL of TRIzol reagent (Thermo Fisher) and 200µL of chloroform. Aqueous fractions were precipitated with 70% ethanol, transferred to silica membrane columns, and purified using the Ambion PureLink^{TM} RNA Mini Kit (Thermo Fisher). RNA was eluted in diethyl pyrocarbonate (DEPC) treated water and samples were quantified by fluorospectrometry (Nanodrop 3000, Thermo Scientific) prior to treatment with DNAse I (Invitrogen) to remove any genomic and plasmid DNA contamination. cDNA was generated from 500 ng of DNase-treated RNA and the Superscript III reverse transcriptase (Invitrogen) from random hexamers (Invitrogen). The resulting cDNA was used as a template for PCR amplification using primers specific to the 5' and 3' regions of the LtxA gene (#39-#32; Table 3). The PCR products were then visualized by agarose-gel electrophoresis.

Codon optimization

The wild-type LtxCA sequence was codon-optimized (CO) using GenScript's OptimumGene™ service (Piscataway, NJ) for expression in an *E. coli* background (see Appendix II). The sequence was divided into two fragments of approximately 1 kb each to separate the complete codon-optimized LtxA (CO_LtxA) region from LtxC and ensure safety during synthesis and handling. Fragment 1 (F1) was cloned into a pAra99a *Hind*III(-) variant using *Kpn*I and *Hind*III and fragment 2 (F2) was then subcloned into pAra99a::CO_LtxAF1 using *Hind*III and *Sal*I. The codon-optimized construct was purified from the EC100 cloning strain and transformed in the *Salmonella* strains as described above for wild-type LtxCABD.

Plate-based hemolysis assay

Columbia blood agar (Accumedia, Neogen Corp.) was prepared according to manufacturer instructions and supplemented with 5% defibrinated sheep's blood (Hardy diagnostics) following sterilization and cooling to 48°C. To test for hemolytic activity, JP2 and VNP20009 were first grown on AAGM and LB respectively at 37°C overnight. Single colonies were then transferred to Columbia agar plates with or without L-arabinose and grown in a 5% CO_2 atmosphere at 37°C for 48 to 72 hours. To induce LtxA expression in VNP20009 strains, Columbia agar was supplied with 200 µg of L-arabinose per plate prior to streaking.

Human cell culture

The human myelogenous leukemia cell line K-562 was obtained from the NIH Division of Cancer Treatments and Diagnostics Repository and ATCC, respectively. Cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with heat-inactivated Fetal Bovine Serum (10% vol/vol; Gibco/Life Technologies, Grand Island, NY), 1% penicillin/streptomycin (10,000 units of penicillin and 10 mg/mL streptomycin; Sigma-Aldrich, St. Louis, MO).

Trypan Blue exclusion assay

THP-1 (ATCC TIB-202) and K-562 (DCTD) were plated at 10^5 cells per mL in 24-well microplates. The cells were treated in triplicate with 0.1 mL of unfiltered VNP20009 supernatants harvested as described above for 30 minutes. After treatment, cells were resuspended to ensure homogeneity and mixed with 0.4% Trypan Blue solution in a 1:1 ratio prior to loading onto a hemocytometer for cell counting. Live (white) and dead (blue) cells were counted in three fields per sample.

#	Primer Sequence (5' – 3')	Anneal Temp (°C)	Primer Enzyme	Vector Enzyme	Description
	WT-LtxA				
1	GATCGGTACCCAGGAGGAATTCACCATGGAGAAAAATAATAATTTTGAAGTGTTAG	61.2	*Kpn*I	*Kpn*I	LtxA F
2	GATCCCCGGGCGCCTCGAGGCGCCGCGGTTAAGCAGTAGTTGCTAACGAATTTGCG	62.6	*Sma*I	*Sma*I	LtxA-CA R
3	GATCCCCGGGGCGCTCGAGGCGATCGATTTAACGTTCTCTTAAACTTTTATCAATAGA	61.9	*Xho*I ClaI	*Sma*I	LtxA-CABD R
4	Ara F1 seq		n/a	n/a	Sequencing; primes in plasmid
5	Trc R2 seq		n/a	n/a	Sequencing; primes in plasmid
6	ACAAAACAGCAAGCTGCACA	60.6	n/a	n/a	Sequencing F1
7	GGTGAAGCACTAGGACAAGTAAAA	59.0	n/a	n/a	Sequencing F2
8	GAATCAGCTCGCCGATAAAA	60.3	n/a	n/a	Sequencing F3
9	CAATAGTTAATGCTGGAGACGG	58.8	n/a	n/a	Sequencing F4
10	GAGAAGGCGATGACCAACTT	59.2	n/a	n/a	Sequencing F5
11	TAACTTTAACTCTTACGCAAATTCGTT	60.0	n/a	n/a	Sequencing F6
12	GCAAATTTTTGCACTGATTACA	58.0	n/a	n/a	Sequencing F7
13	GCGAGTTATGTTGCTGTGAGTT	59.4	n/a	n/a	Sequencing F8
14	CGTTAACCAATCCGGGAA	59.3	n/a	n/a	Sequencing F9
15	CCTGTATCAAATGCACCACG	60.0	n/a	n/a	Sequencing F10
16	TTGCAAAACATACAGTTTTAGATGAA	59.2	n/a	n/a	Sequencing F11

17	AAGACAGGCATGAGAAGC GT	60.0	n/a	n/a	Sequencing F12
18	TTTTGCAGCATCAATATTT TCC	59.1	n/a	n/a	Sequencing R1
19	TTCTTTAATTTATCTCCAA AACTACCA	58.0	n/a	n/a	Sequencing R2
20	GCTTTAATTTTATCGGCGA GC	60.1	n/a	n/a	Sequencing R3
21	GCATTAACTATTGTTGAAC CGGA	60.2	n/a	n/a	Sequencing R4
22	GCCTTCTCCACCGTCAAGT A	60.3	n/a	n/a	Sequencing R5
23	GCTTTACCGGATGAACCA AA	61.2	n/a	n/a	Sequencing R6
24	TCAATGTTGAAAATCCACG G	59.4	n/a	n/a	Sequencing R7
25	GGTTGATCACCATAACGG CT	60.0	n/a	n/a	Sequencing R8
26	AATTCAGAAATAAAATCG TGCG	58.3	n/a	n/a	Sequencing R9
27	TCCACATTGCTAAAGATAG AAACAA	59.2	n/a	n/a	Sequencing R10
28	TTTCAACTTGCATTAAACT CGC	59.4	n/a	n/a	Sequencing R11
29	TTAACGTTCTCTTAAACTT TTATCAAT	55.8	n/a	n/a	Sequencing R12
HlyA C-terminus					
30	TTAGCCTATGGAAGTCA GGGTA	62.5	n/a	n/a	HlyA C-terminus Forward
31	GATCCCCGGGTTATGCT GATGCTGTCAAAGTTA	61.1	*Sma*I	*Sma*I	HlyA C-terminus Reverse
32	GATCCTTAAGAGACAAT TTGAGTTACAGC	55.8	*Alf*II	n/a	LtxA Forward
LtxAΔ60-HlyA					
33	TACCCTGACTTCCATAG GCTAAAAGGCTACTTGA CTTACCTTTA	59.4	n/a	n/a	**Δ**60 HlyA Overlap Reverse
34	TGGACGGAACTCAATAA CTTTG	59.1	n/a	n/a	Sequencing F13
35	CGCCAAAACAGCCAAG CTA	62.3	n/a	n/a	Sequencing R13
LtxAΔ115-HlyA					
36	TACCCTGACTTCCATAG GCTAATTTACCTCGCTG TAACTCAAA	59.9	n/a	n/a	**Δ**115 HlyA Overlap Reverse

37	TGACAGCATCAGCATAACCC	59.7	n/a	n/a	Sequencing F14
38	CTTCTCTCATCCGCCAAAAC	60.0	n/a	n/a	DNA Sequencing F14
RT-PCR					
39	TGACAGCATCAGCATAACCC	59.7	n/a	n/a	5' LtxA amplification F
40	CTTCTCTCATCCGCCAAAAC	60.0	n/a	n/a	5' LtxA amplification R
41	TGACAGCATCAGCATAACCC	59.7	n/a	n/a	3' LtxA amplification F
42	CTTCTCTCATCCGCCAAAAC	60.0	n/a	n/a	3' LtxA amplification R

Table 3. Oligonucleotide primers used in this study.

RESULTS

Heterologous expression of wild-type LtxA in *E. coli* and tumor targeted *Salmonella*

The wildtype LtxCABD operon was amplified by PCR and cloned into the pAra99a multiple cloning site (Figure 5). The pAra99a-LtxCABD sequence was verified by Sanger sequencing of both strands using primers 4-29 (Table 3). The genome of *A. actinomycetemcomitans* strain B5386 (ATCC 29524), which served as the template for LtxA amplification, has not previously been sequenced. Sequence analyses were performed using based on the published HK1651 JP2 genome sequence (Genbank #CP007502.1) to validate the cloned construct. We identified two single nucleotide polymorphisms between the two strains that do not result in amino acid sequence differences; a G to A transition at position 255 and a C to T transition at position 2,562. The B5386 LtxCABD sequence (Appendix I) was deposited to the Genbank database and can be accessed using the following accession number: KY965314.

LtxA expression was first assessed at the mRNA transcript level by RT-PCR using LtxA-specific primers designed to anneal at the 3' and 5' ends of the sequence. This allowed us to verify that the complete LtxA structural toxin mRNA transcript was present. Total RNA was isolated from a Rosetta (DE3) clone carrying the pAra-LtxCABD construct and induced with L-arabinose for 16 hours, as well as from the *A. actinomycetemcomitans* strain JP2, which served as a highly leukotoxic benchmark. The complete LtxA transcript was detected in Rosetta (DE3) as evidenced by the presence of PCR product generated from a cDNA library following treatment with DNase I (Figure 6). The same PCR products are absent when the RNA sample is treated with RNase A prior to the reverse transcription reaction.

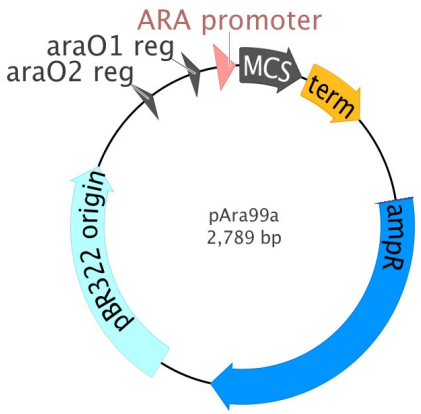

Figure 5. **Plasmid map of the pAra99a vector.** Inserts were introduced into the multiple cloning site (MCS) and expression originated from the L-arabinose inducible promoter (ARA promoter). The vector contains an ampicillin-resistance cassette (ampR) encoding β-lactamase.

To determine whether protein expression is achievable in our background of interest, the tumor-targeted strain of *Salmonella typhimurium* VNP20009, an immunoblot was performed on cytoplasmic fractions prepared from Rosetta (DE3) and VNP20009 induced for 4 hours (Figure 7). An immunoreactive protein signal corresponding to ~115 kDa was detected in the JP2 positive control, as well as in the Rosetta (DE3) and VNP20009 strains, but not from an empty pAra99a vector control. These results suggest that the LtxCABD operon is expressed and that the structural toxin, LtxA, is present in VNP20009 cells.

Secretion of LtxA by VNP20009 by the LtxBD and HlyBD complexes

Having verified LtxA protein expression in the VNP20009 background, we sought to determine whether the protein could be secreted into the culture supernatant. The pAra99a-LtxCA construct was co-expressed with the chloramphenicol- resistance pLG575 plasmid carrying the *E. coli* HlyBD genes (Mackman et al., 1989).

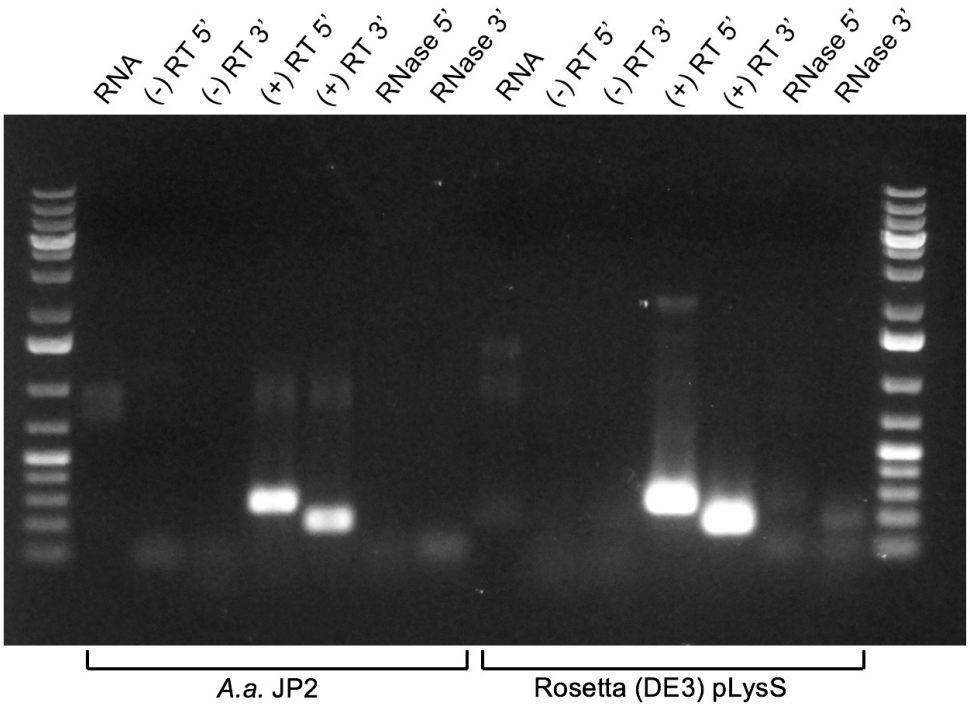

Figure 6. **RT-PCR of JP2 of Rosetta (DE3) pLysS expressing pAra99-LtxCABD**. RNA lanes contain RNA treated with DNase I. (-)RT reactions were treated with 1 uL of DEPC-treated water instead of reverse transcriptase. (+)RT reactions contain cDNA generated from 500 ng RNA. RNase lanes correspond to 500 ng RNA treated with RNase A prior to the RT reaction.

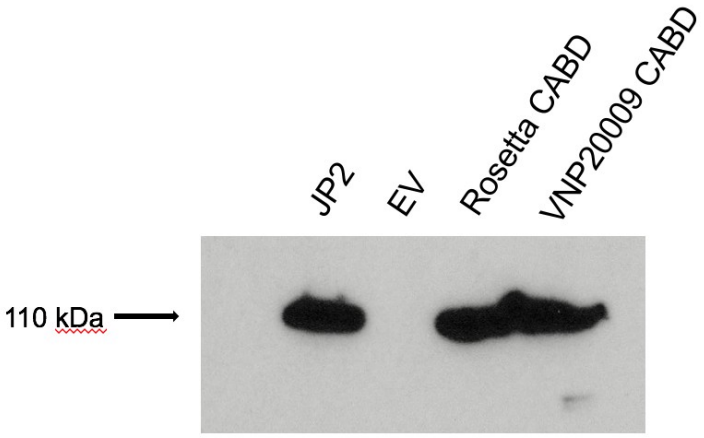

Figure 7. **Anti-LtxA immunoblot of Rosetta (DE3) pLysS and VNP20009 cytoplasmic fraction expressing pAra99a-LtxCABD**. JP2 positive control and pAra99a empty vector negative control.

Immunoblots were performed to determine whether the different clones constructed to optimize secretion by VNP20009 were released into the supernatant. In addition to probing whole pellet fractions, periplasmic and supernatant proteins were precipitated and tested for the presence of LtxA. This allowed us to test whether the LtxBD or HlyBD are functional in VNP20009 and compatible with LtxA translocation compared to LtxCA in the absence of either type I secretion system. A protein signal in the LtxCA-only lane is visible (Figure 8A; lane 3), but appears to be approximately 15 kDa larger than the signal in the LtxCABD lane (Figure 8A; lane 4). Similarly, a ~130 kDA protein is present in the cytoplasm of VNP20009 co-expressing LtxCA and HlyBD (Figure 8; lane 5), and appears in the periplasmic space (Figure 8; lane 5).

Codon optimization of the LtxA sequence

We asked whether LtxCA expression could be optimized for the VNP20009 background. *A. actinomycetemcomitans* is a low GC organism, its genomic content not exceeding 45% (Chen et al., 2009). The LtxA sequence is 37% GC with some regions dropping below 20% (Figure 9). In addition, the LtxA protein sequence contains amino acids encoded by rare codons in *E.* coli and *Salmonella* backgrounds. To address this, the LtxA coding region was codon optimized for expression in *Salmonella* and the GC content of the sequence increased from 37% to just under 50% (Appendix II and III). Immunoblot analysis revealed a lack of protein signal for the codon optimized protein alone or in *trans* with HlyBD (Figure 8; lanes 6 and 7).

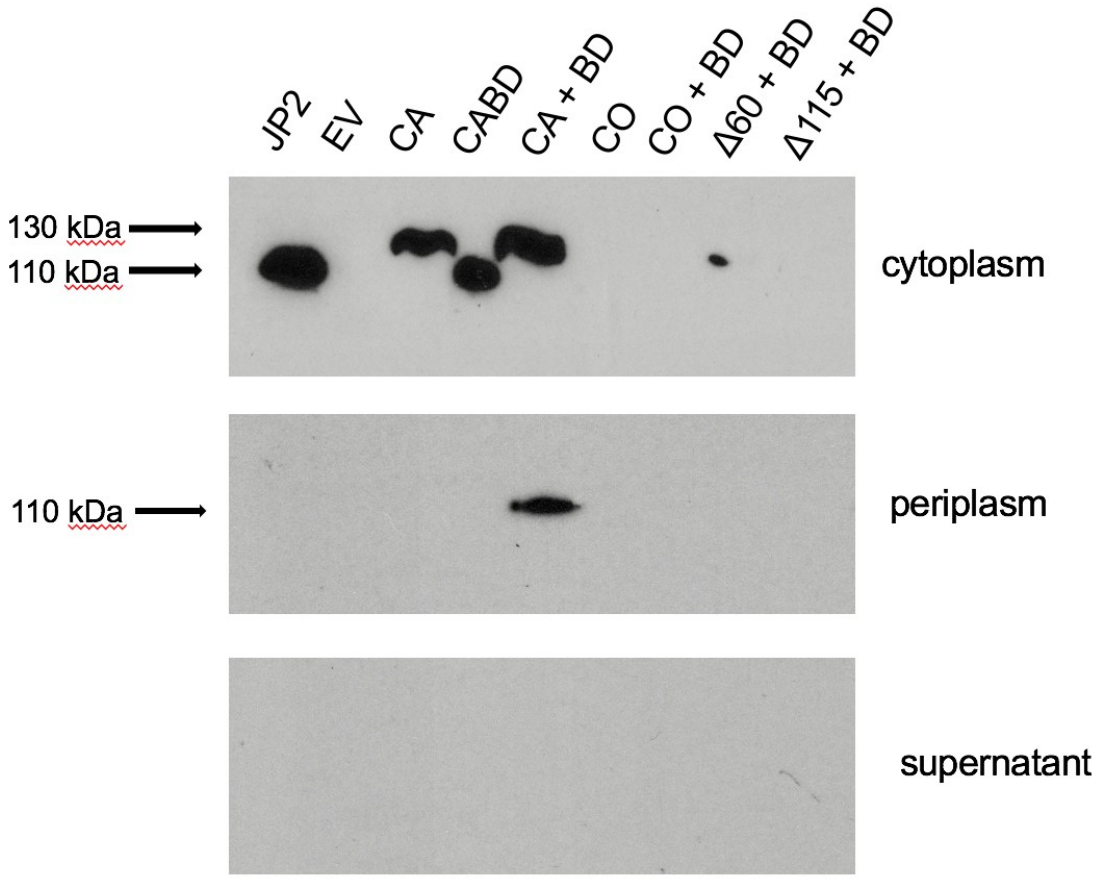

Figure 8. **Detection of the LtxA protein in VNP20009 cytoplasmic, periplasmic, and supernatant protein fractions probed with LtxA antibody.** Anti-LtxA immunoblot comparison of JP2 positive control and VNP20009 strains (1) JP2 positive control; (2) VNP20009 pAra99a-empty vector (EV); (3) VNP20009 pAra99a-LtxCA; (4) VNP20009 pAra99a-LtxCABD; (5) VNP20009 pAra99a-LtxCA + pLG575-HlyBD; (6) VNP20009 pAra99a-CO_LtxCA; (7) VNP20009 pAra99a-CO_LtxCA + pLG575-HlyBD; (8) pAra99a-LtxCA**Δ**60 + pLG575-HlyBD; (9) pAra99a-LtxCA**Δ**115 + pLG575-HlyBD.

LtxA-HlyA C-terminal fusion constructs co-expressed with HlyBD

We then wanted to test whether changing the secretion signal displayed by the LtxA protein could promote secretion by VNP20009. LtxA, but not HlyA, includes stretch of basic residues near the C-terminus of the structural protein, which appears to be responsible for LtxA interaction with the outer membrane (Stanley et al., 1991) and for the protein's relatively high isoeletric point compared to other RTX toxins. This region

also contains an uncleaved signal sequence that mediates interaction with LtxB, LtxD, and TdeA, and thereby controls secretion. The LtxA C-terminus was substituted for the 62-amino acid HlyA equivalent by overlapping extension PCR to facilitate LtxA interaction with the HlyBD/TolC secretion complex and exit the periplasmic space. Because the LtxA C-terminus remains poorly characterized in the literature, two separate constructs were generated to preserve proper protein structure and function. In the first construct, the complete sequence downstream of the *Afl*II site marking the end of the nonapeptide repeats were substituted for the HlyA C-terminus (Figure 10A). In the second C-terminal chimera, only the last 60 amino acids of the LtxA sequence were omitted and replaced for the HlyA secretion signal (Figure 10B).

Expression and secretion of C-terminal fusion LtxA-HlyA proteins was tested by immunoblot and compared to the other LtxA constructs generated in this study. LtxA protein was detected in the cytoplasmic fraction of VNP20009 carrying LtxCA**Δ**60 and HlyBD (Figure 8; lane 8), but not LtxCA**Δ**115 (Figure 8; lane 9). No chimeric protein was detected in the periplasmic or supernatant fractions.

Hemolytic activity of LtxA-expressing VNP20009 strains

A plate-based assay was used to visualize LtxA secretion and cytotoxic activity based on reports that the protein lyses human and sheep erythrocytes when supplied with specific media formulations. Balashova et al. (2006) compared Columbia agar base from several manufacturers and showed that when grown on Columbia agar obtained from Neogen (Accumedia), *A. actinomycetemcomitans* JP2 produced beta-hemolytic colonies. The wildtype, codon optimized, and C-terminal fusion LtxA constructs expressed in VNP20009 with either LtxBD and HlyBD were grown on Accumedia Columbia agar

plates supplied with L-arabinose inducer (Figure 11). The wildtype LtxCABD and LtxCAΔ60 C-terminal LtxA with HlyBD were positive for beta-hemolysis suggesting that even strains that appeared not to produce or secrete toxins as evidenced by immunoblot analyses secrete a hemolytic agent we presume to be active LtxA. Alternatively, the toxin could be released upon lysis off VNP20009 cells within the colony.

Cytotoxic activity of LtxA secreted by VNP20009 against LFA-1 expressing cells

Two human hematopoietic cell lines were used to assess the activity of LtxA constructs against cells expressing LFA-1. An LFA-1 over-expression lineage (THP-1) and an LFA-1 deletion mutant (K-562) were treated with 10% VNP20009 supernatant to cell culture ratio of supernatants containing LtxCAΔ60 + HlyBD, with empty pAra99a vector serving as a negative control. Within 15 minutes of treatment, THP-1 cells showed evidence of increased cell permeability, and by 30 minutes of exposure, complete cell lysis was observed. A Trypan-Blue exclusion experiment (Figure 12) was used to determine percent cell viability at the end of the 30-minute treatment. Nearly all THP-1 cells were unable to exclude the dye, suggesting increased cell permeability and lysis in response to the LtxCAΔ60 toxin, while K-562 cells remained unaffected.

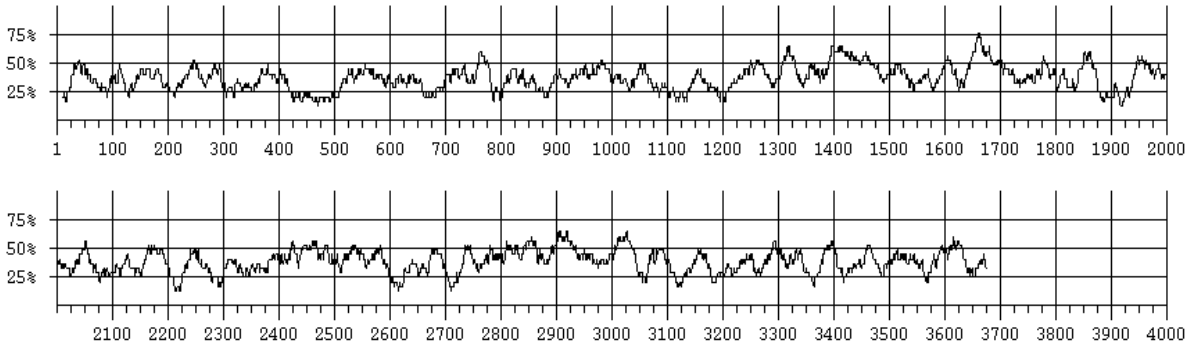

Figure 9. GC plot of the LtxCA sequence. GC content ranged from 19% to 75% and has an average of 37%.

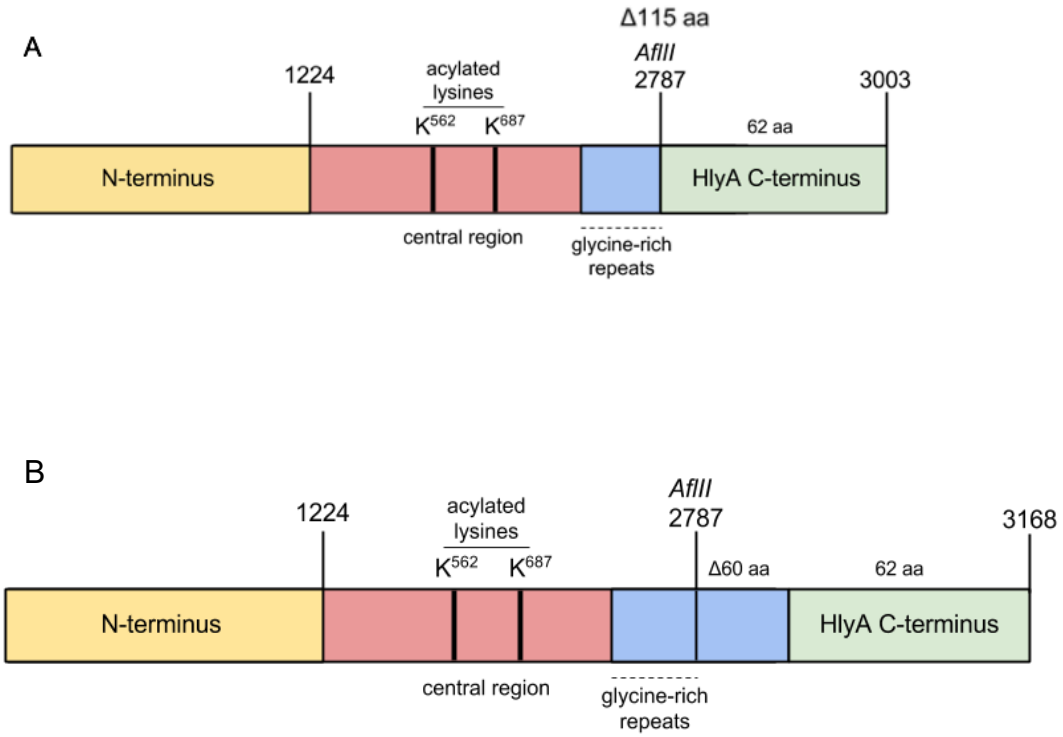

Figure 10. LtxA-HlyA C-terminal fusions generated by overlapping PCR. (A) LtxCAΔ60 lacks the last 60 amino acids which are replaced for the 62 amino acid HlyA C-terminus. (B) LtxCAΔ115 lacks 115 amino acids downstream of the *Afl*II site.

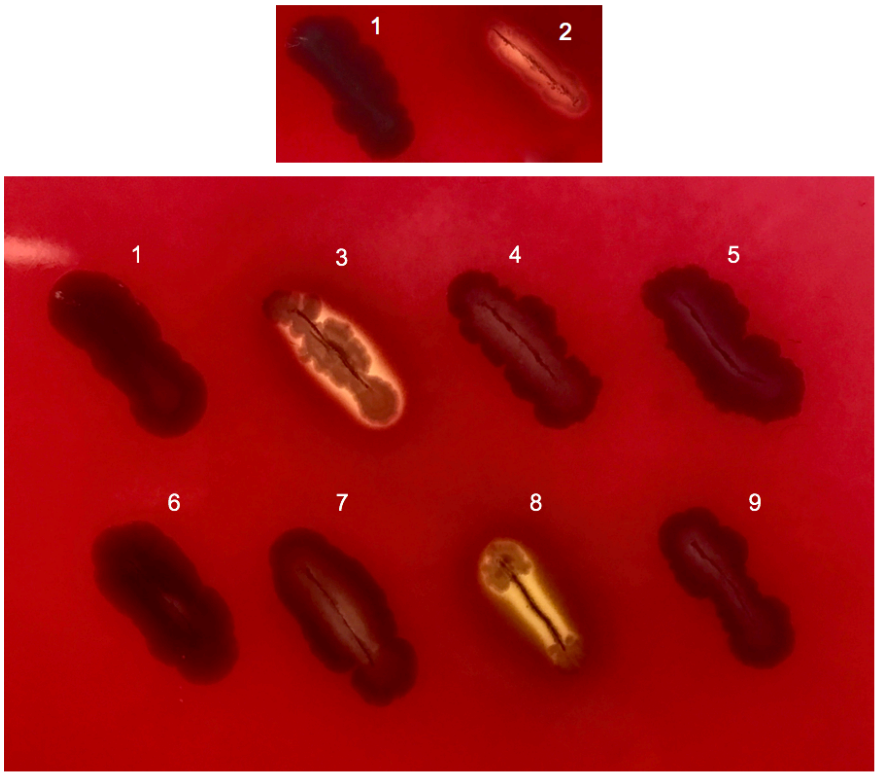

Figure 11. **Columbia blood agar plate-based hemolysis assay of VNP20009 strains** on plates supplied with no arabinose and 200 µg L-arabinose. (1) VNP20009 pAra-EV negative control; (2) JP2 positive control; VNP20009 (3) pAra99a-LtxCABD; (4) pAra99a-LtxCA + pLG575-HlyBD; (5) pAra99a-LtxCA; (6) pAra99a-CO_LtxCA; (7) VNP20009 pAra99a-CO_LtxCA + pLG575-HlyBD; (8) pAra99a-LtxCA**Δ**60 + pLG575-HlyBD; (9) pAra99a-LtxCA**Δ**115 + pLG575-HlyBD.

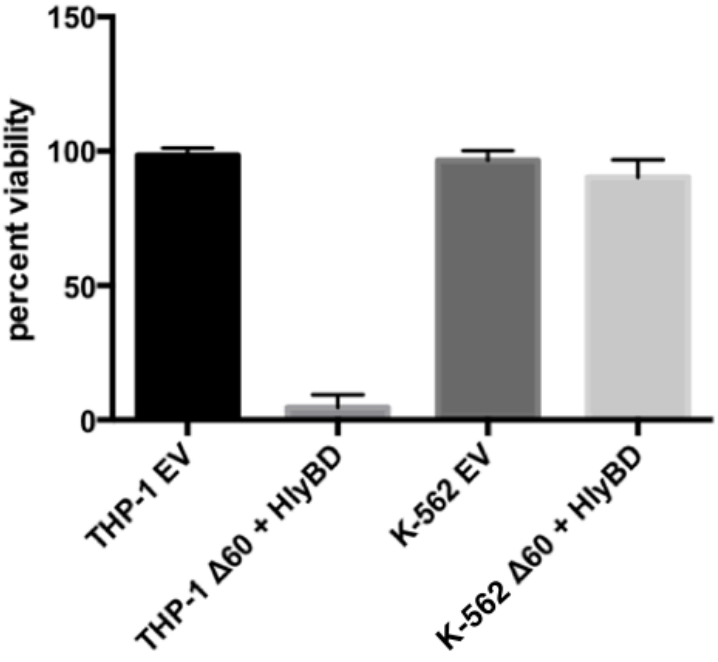

Figure 12. **Trypan Blue-exclusion cytotoxicity assay of THP-1 (LFA-1 +) and K-562 (LFA-1 -).** Cells were stained with Trypan-Blue in three fields from triplicate wells treated with $1/10^{th}$ culture volume of VNP20009 LtxCAΔ60 + pLG575-HlyBD supernatants for 20 minutes. Percent viability was calculated from the number of dead cells divided by the number of live cells per field. Bars represent SEM.

DISCUSSION

Summary of results

The objective of this study was to evaluate the antitumor potential of *A. actinomycetemcomitans* LtxA when expressed and secreted by the tumor-targeted *Salmonella typhimurium* strain VNP20009. The LtxA encoding genes were first cloned into the arabinose-inducible vector pAra99a. We selected this plasmid vector for our studies because of its inducible nature, which allows for stringent control of toxin expression based on the presence of the inducer. We have previously demonstrated its use as a heterologous expression vector for other toxic proteins, namely *Pseudomonas* ToxA-TGFα chimeras (Quintero et al., 2016).

In previous work by Kraig et al. (1999), the LtxA operon was cloned for expression from its own promoter in various *E. coli* strains. Toxin expression and activity were validated solely based on the results of experiments in which human cell lines expressing LFA-1 were exposed to cell lysates presumed to contain the toxin. Based on these accounts, we transformed the pAra99a-LtxCABD construct in *E. coli* BL-21 strain Rosetta (DE3) carrying the pLysS plasmid, which encodes codons with low usage in the *E. coli* background. Here, we showed that LtxA could be expressed both at the mRNA and protein levels in BL-21 and *Salmonella*.

Early studies of LtxA secretion by *A. actinomycetemcomitans* and *E. coli* expressing LtxA reported that the toxin remained associated with the outer membrane and was not readily released into the culture medium (Tsai et al., 1984). Later, Kachlany et al. (2000) reported that the toxin is in fact secreted into the culture medium and attributed the initial reports to poor isolation methods and sub-optimal culture conditions

(Kachlany et al., 2000). Although it is now widely accepted that all *A. actinomycetemcomitans* strains secrete active LtxA into the culture medium, the toxin may be inefficiently transported out of the periplasmic space by *Salmonella* outer membrane channel protein TolC due to interaction incompatibilities with the LtxBD complex (Figure 13B). We hypothesized that this could be overcome by replacing the complex through which LtxA exits the cell for one known to be functional in *Salmonella*; the *E. coli* hemolysin BD (HlyBD) complex (Figure 13C). Many proteins have been expressed and secreted using the HlyBD system with reported success, including biodegradable plastic precursors (Rahman et al., 2013) and foreign bacterial toxins (Blight & Holland, 1994). We constructed C-terminal chimeras of LtxA and HlyA to promote interaction between the LtxA protein and the HlyBD secretion complex (Figure 13D).

Immunoblots using a monoclonal anti-LtxA antibody were performed to detect LtxA protein in VNP20009 cellular fractions. However, we failed to obtain consistent results for the clones tested. In some instances, no protein product was obtained, and in others, different band sizes were observed. Larger LtxA protein variants ranging from 130 to 180 kDa have been reported in the literature and the presence of multiple LtxA protein products has been attributed to several factors. Ohta et al. (2001) showed that the LtxA protein readily binds to nucleic acids and that this interaction has a functional role in tethering the toxin to the bacterial cell surface. The presence of nucleic acids in the pellet lysates prepared in this study may contribute to poor solubilization of LtxA or to inconsistencies in resolving the protein by electrophoresis. We conclude that immunoblot analysis may not be suitable for the detection of LtxA protein without protocol alterations

to disrupt its interaction with nucleic acids.

Because we could not rely on immunoblots to assess whether LtxA constructs were functionally expressed and secreted by VNP20009, a separate approach was used based on a report by Balashova et al. (2006) that LtxA confers beta-hemolytic activity to colonies when grown on Columbia agar obtained from Neogen (Accumedia). VNP20009 clones expressing the LtxA constructs were grown on Accumedia Columbia agar supplemented with L-arabinose inducer, and our results suggest that both VNP20009 expressing the wildtype complete LtxCABD construct and LtxCA**Δ**60 + HlyBD lyse erythrocytes and thus produce active toxin. These findings first indicate that the presence of a BD inner membrane complex is required for toxic activity, likely because of its role in mediating secretion of the toxin into the extracellular space. Second, the results suggest that LtxCA and HlyBD are not compatible, as LtxCA does not produce a hemolytic or cytotoxic effect even when co-expressed with HlyBD. Finally, we propose that a stretch of approximately 50 amino acids downstream of the glycine-rich repeats region (marked by an *Afl*II site at position 2787) is required for proper LtxA function, as the LtxCA**Δ**115 C-terminal fusion did not appear to be hemolytic in Columbia agar experiments.

The LtxA gene is low in GC percentage, a concern for heterologous expression because AT-rich genes may be inefficiently transcribed or silenced by a *Salmonella* host as they possess mechanisms to protect against the expression of such sequences. There is evidence to suggest that repression by histone-like nucleoid structuring protein (H-NS) of AT-rich sequences may protect the host organism from the possible deleterious effects of foreign DNA expression (Ali et al. 2014). Therefore, we synthesized a codon-optimized

LtxA sequence in which AT-rich regions were improved to promote efficient expression. We did not detect CO protein in immunoblot or Columbia agar experiments, and attribute this results to the disruptions in AT-rich regulatory regions that may be required for proper toxin expression and function.

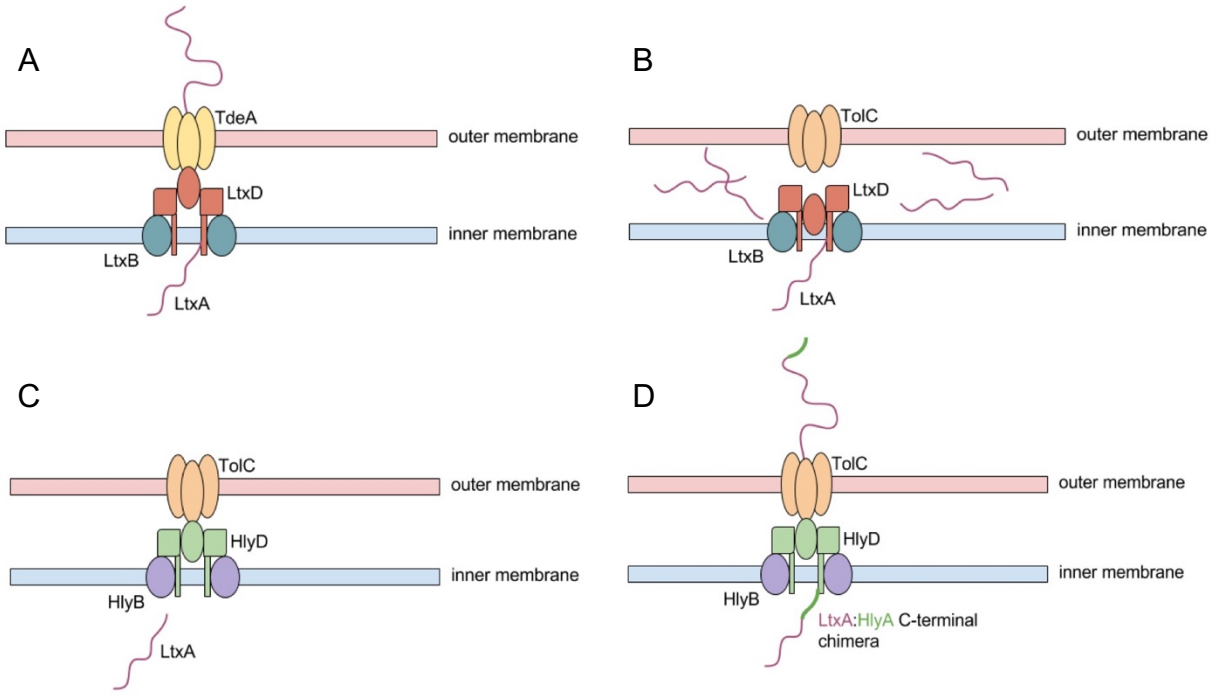

Figure 13. Hypothesized secretory incompatibilities between LtxA and the VNP20009 membrane system. (A) LtxA is secreted by its own LtxBD/TdeA machinery in *A. actinomycetemcomitans*. (B) In *Salmonella*, LtxBD may not interact with TolC, resulting in LtxA accumulation in the periplasmic space. (C) LtxA is co-expressed with HlyBD in *Salmonella*, but LtxA may not recognize the HlyBD complex. (D) A C-terminal LtxA- HlyA chimera can interact with HlyBD and be secreted via HlyBD/TolC

The LtxA-HlyA C-terminal fusion constructs generated in our experiments showed the strongest hemolytic activity when co-expressed with HlyBD, with LtxCAΔ60 consistently having the most rapid and pronounced effect. We therefore wanted to test whether it was cytotoxic against human hematopoietic cells lines with published

sensitivity to LtxA. The chimeric toxin rapidly permeabilized and lysed THP-1, a LFA-1 over-expression leukemia lineage, while having no effect on K-562, a LFA-1 negative leukemia cell line. These results are in keeping with previous studies reporting rapid lysis of LFA-1 expressing cells (Kachlany et al., 2011) as well as target specificity, evidenced by a lack of effect on LFA-1 negative lines. A key difference in our experiments is the use of VNP20009 culture supernatants with no LtxA purification step. Future experiments will determine the toxic activity and specificity of the other LtxA constructs generated in this study.

Significance

Despite the development of numerous treatment options for cancer patients, conventional therapies fail to prevent tumor recurrence. In many instances, drug-resistance, relapse, and metastasis can occur. Even in cases where chemotherapy and radiation possess potent anticancer properties, they are often toxic to normal tissues. The development of effective and safe treatments must therefore address two fundamental aspects of cancer therapy; cancer-killing efficacy and target specificity. Tumor-specific bacterial vectors have the potential to address both problems, as well as go beyond the primary tumor and extend their effects to metastatic tumor sites, which are notoriously resistant to conventional treatments.

Salmonella strain VNP20009 has an intrinsic ability to selectively colonize and contribute to tumor growth delay in murine models. A clinical trial using VNP20009 on terminally ill metastatic melanoma patients indicated that *Salmonella*'s ability to cause tumor inhibition that was observed in mice did not occur in humans, but the bacteria safely colonized some tumors (Toso et al., 2002). The lack of tumor inhibition observed

in humans is not fully understood, although it is plausible that the attenuation that characterized this strain hinders its ability to evade an immune response.

We have recently demonstrated that cancer cell killing by VNP20009 can be engineered through heterologous expression of cytotoxic proteins that target cancer cells overexpressing epidermal growth factor receptor (EGFR; Quintero et al., 2016). In this study, we explored the cell killing potential of *A. actinomycetemcomitans* LtxA in a tumor-targeted *Salmonella* background. This would represent a means for targeted delivery of a cancer cell killing toxin to highly invasive tumor variants, while relying on the system's demonstrated safety in animal models and human clinical trials to avoid toxicity. The therapeutic potential of purified LtxA in the treatment of hematological malignancies has already been demonstrated (Kachlany et al., 2010, DiFranco et al., 2015). However, several concerns remain regarding the potential off-target effects when the purified toxin is administered to human patients. Here, we have investigated employing a highly targeted bacterial system to be expressed and secreted to have the potential to selectively deliver the toxin to tumor sites and overcome potential interaction with healthy white-blood cells essential for proper immune function. Delivery of LtxA directly to a tumor or lymphoma in the VNP20009 background has the potential to address the limitations of using purified LtxA as a therapeutic agent.

The similarities between bone marrow-derived cells (BMDCs) and metastatic cancer cells are numerous and well documented (Pawelek & Chakraborty, 2008; Yilmaz et al., 2005). There is evidence that metastatic cancer cells are the result of fusion events between non-metastatic primary tumor cells and BMDCs such as leukocytes and macrophages (Pawelek & Chakraborty, 2008). The high homotypic fusion capacity of

tumor cells and BMDCs, as well as evidence for their direct contact in tumor environments, led researchers to consider fusion as a source of metastatic conversion over a hundred years ago (Aichel, 1911). Since then, BMDC- cancer cell hybrids have been characterized in humans and animals (Yilmaz et al., 2005). *In vitro* experiments have demonstrated that melanoma cells that acquire macrophage-specific functions through artificial fusion, such as the secretion of metalloproteases, expression of pro-migratory molecules, and the ability to stimulate angiogenesis (Powell et al., 2011), have greater metastatic potential when injected into mice than do primary cancer cells alone (Larizza & Schirrmacher, 1984). In addition, it has been reported that metastatic T-cell lymphoma cells overexpress the macrophage-specific Mac-1 antigen (Larizza et al., 1984). Regardless of whether metastatic cells acquire these properties via fusion events or by alternative processes, such as trans-differentiation (Wicha et al., 2006) or epithelial-to-mesenchymal transition (EMT) (Clawson, 2013), LtxA may have the intrinsic ability to target metastatic cells of hematological and non-hematological origin due to its high degree of specificity for human β_2-integrins.

In addition to the toxin's specificity to cells expressing β_2-integrin, LtxA was recently shown to interact with sialic acid residues found on host cell membrane glycoproteins and glycolipids. Munksgaard et al. (2014) showed that sialic acid is required for the lysis of human erythrocytes and cell lines expressing β_2-integrins by LtxA. This suggests that sialic acids play significant role in mediating LtxA cytotoxic activity, which has been hypothesized to be caused by the negative charge conferred to the membrane surface by these residues. Notably, tumor cells that display hypersialylation, or increased expression of sialic acid on membrane glucoconjugates, are

linked to poor prognosis in cancer patients (Büll et al., 2014). It is possible that heterologous expression of LtxA by a tumor-targeted *Salmonella* could selectively kill hypersialylated cancer cell variants, further increasing the system's efficacy and target specificity for high risk tumor cell variants.

Future directions

Future experiments include further enhancing LtxA activity in the *Salmonella* supernatant by co-expressing the toxin along with protease inhibitors. Tumors secrete a variety of proteases, which facilitates cell escape from the primary tumor mass, extravasation, and the establishment of secondary tumors during metastasis. A consequence of this is the risk of proteolytic degradation of toxic proteins designed to eradicate cancer cells. Our lab has shown that toxic proteins can be co-expressed with sunflower trypsin inhibitor (SFTI) in the VNP20009 background to prevent proteolytic degradation of these toxins when secreted by the tumor-targeted vector, thereby protecting their cytotoxic activity and enhancing their efficacy (Quintero et al., in submission).

Due to the high degree of genetic variation that characterizes tumor cell populations, it is unlikely that a single targeted compound will completely eradicate the entirety of a tumor mass and affect high-risk cell types, namely cancer stem cells and/or pro-metastatic cells. Combining this approach with others currently being developed for use in this system would allow targeting multiple cell types with variable metastatic potential to eradicate the entire tumor volume rather than a subset of tumor cells. Including a toxin that is inherently specific to properties displayed by these cell types has the potential to increase the system's therapeutic potential and extend its effects to areas

that remain inaccessible by current treatment options. To explore this, combination studies including LtxA could be performed using other tumor-targeted toxins developed by members of the Bermudes lab such as the EGFR-specific *Pseudomonas* ToxA chimeras (Quintero et al., 2016), cytolethal distending toxins (CLDTs; Vincent et al., 2017), and *E. coli* cytotoxic necrotizing factor-1 (CNF-1).

Finally, the proposed approach must demonstrate safety in animal models to advance to the clinic should it prove successful in early stages of development. In its current form, the toxin is relatively specific to human and non-human primates but can also kill rodent white blood cells expressing murine LFA-1 (Kachlany et al., 2014), and therefore safety can be tested in Rhesus macaque and potentially in mice. Alternatively, the species specificity of LtxA could be altered by modifying the repeats region of the toxin, which is responsible for conferring species as well as cell-type specificity. This would allow testing of the system in other model organisms. For future studies in animals, the constructs would be inserted into the chromosome of VNP20009 expressed from a single copy of the operon using the lamba red recombinase system (Wanner et al, 2000) to achieve genetic stability and lack antibiotic resistance during evaluation of cytotoxicity of LtxA protein.

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APPENDIX I. *A. actinomycetemcomitans* strain CDC B5386 LtxCABD sequence

LtxC LtxA LtxB LtxD

Ribosome binding sites start codon stop codon SNP

ataaattattaaca**aggaga**tttaatATGGAGAAAAATAATAATTTTGAAGTGTTAGGGTATGTGGCTTGGT
TATGGGCAAATTCTCCTTTGCATAGAAATTGGTCTTTATCATTACTTGCTATTAATGTACTGCCTGCAATTC
AATATGGGCAATATACATTATTAATGAGAGATGGGGTTCCTATTGCTTTTTGTAGCTGGGCAAATTTAAGCC
TTGAGAATGAGATAAAATATCTTGAAGATGTATCATCTCTTGTTTATGACGACTGGAATTCCGGTGATCGTA
AATGGTTTATTGATTGGATTGCGCCATTTGGTCACAATTATGTGCTTTATAAACATATGCGTAAATCATTTC
CCTATGATTTATTCAGATCAATTCGTGTTTATAAAGGTTCGTCAGAGGGCAAAATTACTGAATTCCATGGAG
GTAAAGTCGATAAACAATTAGCTAATAAAATCTTTCAACAATATCATTTTGAGTTAATCAATGAATTAAAAA
ACAAATCTGAAGTTATATCTATTAATTA**Aggagaa**tagattATGGCAACTACTACACTGCCAAATACAAAAC
AGCAAGCTGCACAGTTTGCAAATTCAGTTGCAGATAGAGCTAAGGAAAATATTGATGCTGCAAAAGAACAAT
TGCAAAAGGCGTTAGATAAATTAGGGAAGACAGGTAAGAAATTAACTTTATATATCCCTAAGAATTACAAAA
AAGGAAATGGTCTTACTGCGCTTATAAAAGCAGCACAGAAGTTAGGGATTGAAGTATATCATGAAGGGAAAG
ACGGCCCAGCATTAACTAATGGTATTTTAAATACTGGGAAAAAATTACTTGGTCTTACCGAACGAGGTTTAA
CTTTATTTGCTCCGGAATTAGATAAATGGATTCAAGGTAATAAACATTTAAGTAATTCTGTGGGTAGTACTG
GAAATTTGACAAAAGCGATAGATAAGGTTCAGAGTGTTCTTGGTACGTTACAAGCGTTTTTGAACACCGCAT
TTTCGGGCATGGATTTAGATGCCTTAATTAAAGCCCGTCAAAATGGTAAAAATGTAACAGATGTACAGCTAG
CAAAAGCCAGTCTTAACCTGATTAATGAATTGATTGGTACTATTTCTAGCATTACAAATAATGTAGATACTT
TTTCTAAACAACTTAATAAGTTAGGTGAAGCACTAGGACAAGTAAAACATTTTGGTAGTTTTGGAGATAAAT
TAAAGAATTTACCTAAGTTAGGTAATCTTGGAAAAGGTTTAGGTGCATTATCCGGTGTATTGTCGGCTATAT
CAGCGGCTCTATTACTTGCAAATAAAGATGCTGATACTGCAACGAAAGCAGCGGCTGCAGCTGAATTGACAA
ATAAAGTGCTAGGTAACATCGGTAAAGCGATCACACAATACTTGATTGCTCAACGTGCTGCAGCGGGGCTTT
CTACTACGGGACCTGTCGCAGGGTTAATTGCCTCTGTGGTCAGCTTGGCAATCAGCCCTTTGTCTTTCCTAG
GTATTGCGAAACAATTTGATCGTGCGAGAATGCTTGAGGAATACTCGAAACGCTTTAAGAAATTTGGTTATA
ACGGCGATAGTTTACTTGGTCAATTCTACAAAAATACAGGGATCGCAGATGCTGCGATTACAACGATTAACA
CTGTATTAAGTGCTATTGCAGCAGGGGTTGGTGCAGCCTCCGCCGGTTCTTTAGTTGGTGCGCCAATCGGTT
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GAAAAATATTACTTTAGATGCGATTGAACATCCAAAGCTTGGACTTGTATTTAATACGATTATTGAATTGGA
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GACAGGCATGAGAAGCGTTATAAGCTATCTACTTAGCCCATTAGAAGAATCTATTGATAAAAGTTTAAGAGA
ACGTTAA

APPENDIX II. CODON AND GC% OPTIMIZATION

Fragment 1

Codon Adaptation Index (CAI)

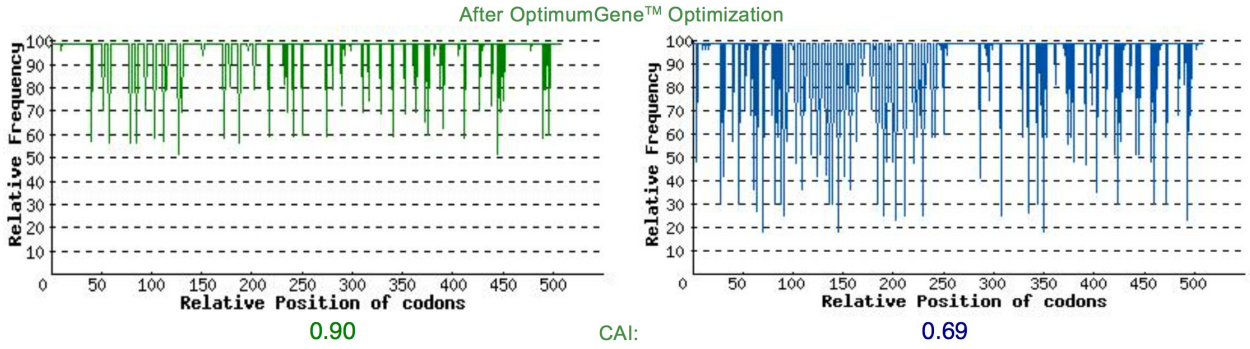


Figure 1a. The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level.

Frequency of Optimal Codons (FOP)

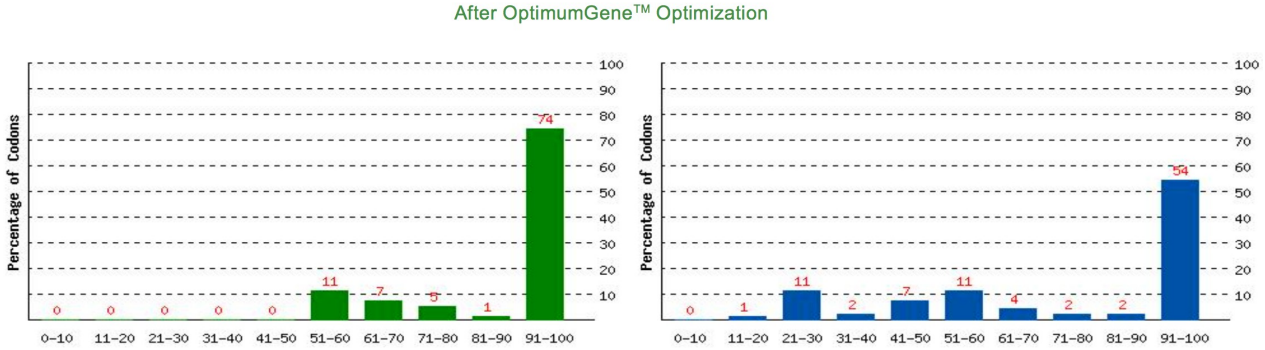


Figure 1b. The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

GC Content Adjustment

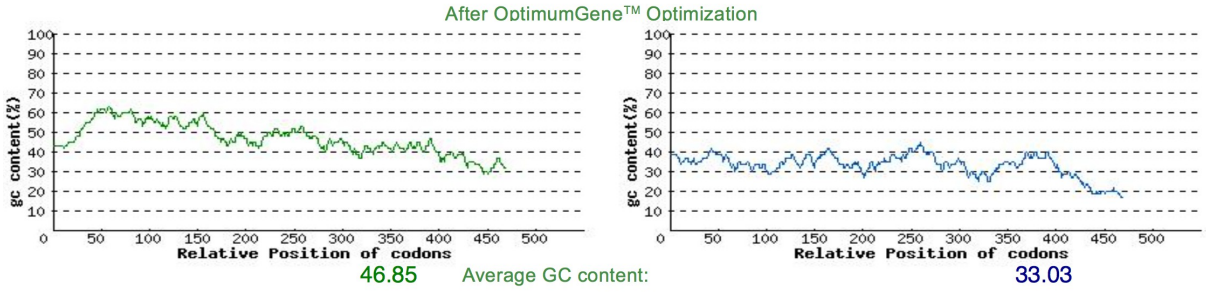
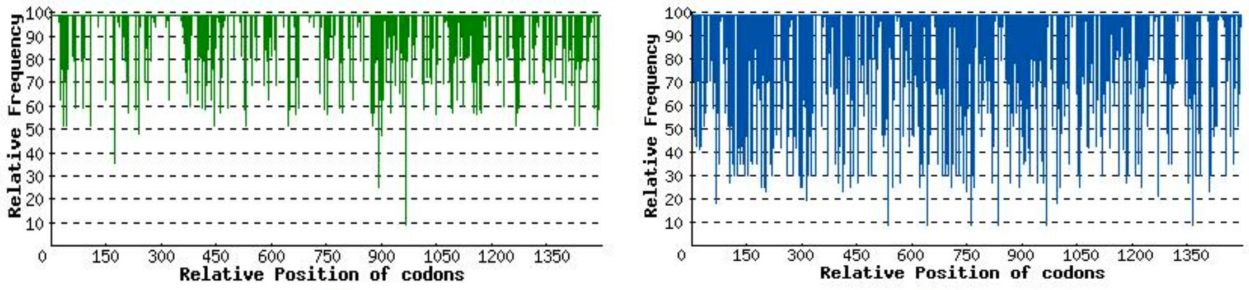


Figure 2. The ideal percentage range of GC content is between 30-70 %. Peaks of %GC content in a 60 bp window have been removed.

Fragment 2

Codon Adaptation Index (CAI)

After OptimumGene™ Optimization



0.87 CAI: 0.65

Figure 1a. The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level.

Frequency of Optimal Codons (FOP)

After OptimumGene™ Optimization

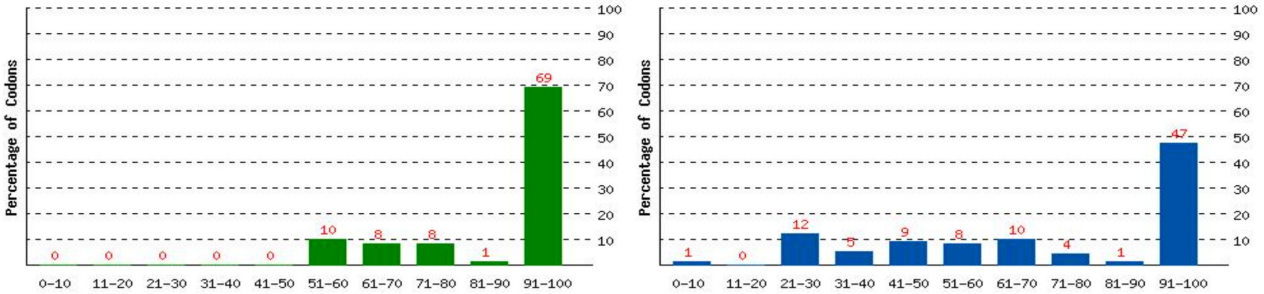
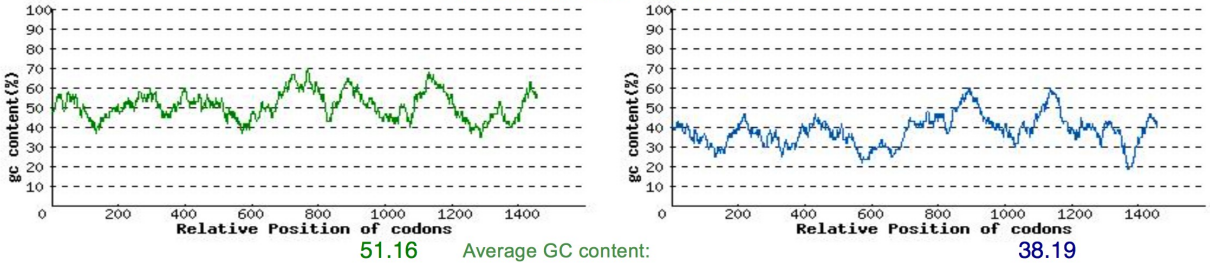


Figure 1b. The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

GC Content Adjustment

After OptimumGene™ Optimization



51.16 Average GC content: 38.19

Figure 2. The ideal percentage range of GC content is between 30-70 %. Peaks of %GC content in a 60 bp window have been removed.

APPENDIX III. Codon Optimized LtxA Sequence

*Kpn*I start

GGTACCCAGGAGGAATTCACCATGGAAAAAAATAACAACTTTGAAGTGCTGGGCTATGTGGCTTGGCTGTGGGCAAACTC
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GCAAACTCCCTGGCAACCACCGCCTAAGACGTCgcgCCTAGGgcgGTCGAC

stop *Aat*II *Avr*II *Sal*I

AAGCTT = *Hind*III