Development of contamination resistance strategies for bioindustrial applications

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Bioresource Technology

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Abstract

Contamination by lactic acid bacteria is a major source of inefficiency in the bioethanol industry. Contaminated fermentations exhibit lowered ethanol yields and contamination events often require shutdown of the entire process for cleaning and decontamination. The first objective of this study was to develop fermentation yeast that secrete bacteriocins, proteins that kill lactic acid bacteria, to counteract this contamination. A strain that secretes leucocin A was successfully developed; however, the strain did not exhibit secretion levels that were industrially relevant. Two reasons for poor secretion: the interaction of the bacteriocin with the cell membrane and the prevention of diffusion by the cell wall, were established. The second objective of this study was to produce the circular bacteriocin, carnocyclin A, heterologous in *E. coli*, with the ultimate goal of producing industrial levels of the bacteriocin for use in the food and biorefining industries. The bacteriocin was produced but was not cyclized at detectable levels. The third objective of the study was to screen for additive and synergistic properties of antimicrobials used in the bioethanol industry against lactic acid bacteria isolated from industrial ethanol production facilities. The antimicrobials screened included a conventional antibiotic, a hop compound, and two bacteriocins. The antimicrobials exhibited additive properties, with one leucocin A potentially showing synergistic properties with other inhibitors including nisin.

The development of new biological fermentation pathways for yeast, as well as engineering yeast to produce proteins like bacteriocins, could be expedited by improving cloning processes. The fourth objective of this study was to develop a ligation independent cloning methodology to improve the workflow of the cloning process and the percentage of positive clones produced. This methodology had similar percentage positive clones to other cloning processes.

Preface

This thesis is the original work of Erin Dul. The research in this thesis was conducted in collaboration with members of the Biorefining Conversions and Fermentation Laboratory led by Dr. David Bressler at the University of Alberta. I designed and performed experiments, and interpreted and discussed results under the guidance of Dr. Lynn McMullen (Chapter 2, Chapter 4), Dr. David Stuart (Chapter 2, Chapter 5), and Dr. David Bressler (all chapters). Discussion of results, revisions, and editing of the manuscripts was supported by Dr. Michael Chae (all chapters).

The plasmid YCplac111-P_{GAL1} (Chapter 2) was provided by the Stuart Lab, Department of Biochemistry, University of Alberta.

MALDI-MS (Chapter 3) was performed by technical staff at the Mass Spectrometry Facility, Department of Chemistry, University of Alberta. Tryptic digest and LC/MS (Chapter 3) was performed by technical staff at the Institute for Biomolecular Design, Department of Biochemistry, University of Alberta.

Acknowledgements

Thank you to my supervisor, Dr. David Bressler. I learn something every time I am in the same room as you. Thank you for your leadership, patience, encouragement, and the opportunity to experiment and learn. Thank you to my supervising committee members, Drs. Lynn McMullen and David Stuart, for your thoughtful suggestions and reassurance throughout this process. Thank you to Dr. John McCormick, Dr. Michael Gaenzle, and Dr. Dominic Sauvageau, members of my examining committee, for your insight.

Thank you to Dr. Michael Chae for the very thoughtful critique on the drafts of this manuscript. I feel so fortunate to have benefitted from your knowledge, experience, and careful eye for detail. Thank you to other members of the leadership team in the Bressler lab who have offered advice throughout the research process: Drs. Natisha Stashko, Paolo Mussone, and Archana Parashar. Thank you to my fellow graduate students: Isabel, Tizazu, Justice, Koel, Yiqiong, Emily, Michael, Pooran, Yuko, and Asima. I have learned so much from all of you and I am grateful to have shared this experience with you. Thank you to Jingui Lan, Garson Law, and Allie MacDonell Whiddon for the technical support.

I have benefitted from the mentorship, thoughtful advice, and friendship from members of the broader university community throughout this process and I would like to thank Kelly Maher, Ange Scott, Chris Dambrowitz, Dominic Sauvageau, Shaun McKinnie, Melissa Haveroen, Pamela Bentley, Heather Vandertol-Vanier, Nancy Bray, Nikki van Dusen, Owen Scadeng, and Matt Schellenberg for all of the advice and encouragement.

This research, as well as the numerous professional development opportunities and travel that I have experienced, was possible thanks to the financial support provided by NSERC, Alberta Innovates Technology Futures, the University of Alberta, the Biorefining Conversions Network, and BioFuelNet.

Thank you to all of my family and friends, but especially Jay, Mom, Dad, and Kathy. You have been so patient, loving, and supportive throughout this process.

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List of Symbols and Abbreviations

6His	His-His-His-His-His-His
AU	arbitrary unit
APT	All Purpose Tween
C.	*Carnobacterium*
CarnoA	carnocyclin A
CFU	colony forming units
CLSI	Clinical Laboratory Standards Institute
CM	Complete Minimal
CM-Leu	Complete Minimal media – leucine
°C	degrees Celsius
D.	*Dekkera*
DDG	dried distillers grains
DDGS	dried distillers grains and solubles
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DTT	dithiotheritol
E.	*Escherichia*
FIC	fractional inhibitory concentration
fmol	femtomole
FSMA	Food Safety Modernization Act
g	gram
g	gravitational force
GFP	green fluorescent protein
h	hours
IU	inhibitory unit
kb	kilobase
kDa	kilodalton
kg	kilogram
L	liters

L.	*Leuconostoc*
LAB	Lactic Acid Bacteria
La.	*Lactobacillus*
LB	Luria-Burtani
Lc.	*Lactococcus*
LC-MS	liquid chromatography-mass spectrometry
LEU	leucocin A
LIC	ligation independent cloning
M	molar
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MIC	minimal inhibitory concentration
min	minutes
mg	milligram
mL	milliliters
mM	millimolar
nm	nanometers
nt	nucleotide
OD_{600}	Optical Density at 600 nm
PCR	polymerase chain reaction
pI	isoelectric point
PMSF	phenylmethanesulfonylfluoride
ppm	parts per million
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SICLOPPS	split intein circular ligation of peptides and proteins
TCA	trichloroacetic acid
TCEP	tris(2-carboxyethyl)phosphine
U	unit
UHP	urea-hydrogen peroxide
U.S.	United States

USFDA	United States Food and Drug Administration
USDA	Unites States Department of Agriculture
v/v	volume per unit volume
YC_p	yeast centromeric plasmid
YI_p	yeast integrating plasmid
YP	Yeast Extract Peptone
YP-gal	Yeast Extract Peptone + galactose
YP-glu	Yeast Extract Peptone + glucose
YP-raff	Yeast Extract Peptone + raffinose
WDGS	wet distillers grains
w/v	weight per unit volume
Σ	sum
$	dollars
μL	microliter

Chapter One: Introduction and Literature Review

1.1 Bioethanol

Ethanol is one of the earliest products of biotechnology; humans have used yeast to convert biomass to ethanol for thousands of years to produce beverages. Ethanol has been used as a fuel since the late 19th century; however, use of bioethanol fell out of favor after petroleum gained popularity as a fuel source. Recent environmental and economic policies have driven the increase in production of bioethanol in the 21st century. The gross value of the output of the bioethanol industry in the U.S. in 2013 was estimated to be $44 billion (Renewable Fuels Association, 2014a).

1.1.1 First generation bioethanol

First generation biofuels are conventional biofuels derived from food crops. In North America, first generation bioethanol is derived mainly from corn, but wheat is used in some facilities (Evans, 2013; Renewable Fuels Association, 2014a). There are two distinct processes for bioethanol production that differ based on the treatment of the grain prior to fermentation: the dry-milling process and the wet-milling process.

Dry-milling typically proceeds through eight steps: 1) delivery of the grain to the fermentation plant; 2) cleaning the grain of debris and ground into a course flour; 3) soaking of the grain and treatment with α-amylase to liquefy the starch, jet-cooked, and again soaked and treated with α -amylase and glucoamylase (alpha-amylases break down the starch into shorter polysaccharides, glucoamylase releases glucose molecules from starch) in order to release sugars from the starch; 4) yeast is added and simultaneous saccharification and fermentation occurs, the glucoamylase enzyme continues to release sugars from the starch while the fermentation yeast, typically *Saccharomyces cerevisiae*, converts the sugars to ethanol; 5) distillation to a 95% (v/v) ethanol/water ratio; 6) dehydration by molecular sieve to remove the remaining water; 7) ethanol denaturation and storage; and 8) co-product recovery (S. Lee & Shah, 2013).

In wet-milling, the individual components of the grain are separated prior to fermentation. There are eight steps to the wet-milling process: 1) the grain is delivered,

inspected, and stored; 2) the grain is steeped at 50°C for 24-48 h to swell the grain; 3) the germ is separated by coarse crushing followed by cyclone separation, oil is extracted from the germ; 4) the remaining contents are ground and the fiber is removed by filtration; 5) the starch is separated from the protein by centrifugation; 6) the starch is enzymatically or chemically converted to syrup; 7) the glucose syrup is fermented to ethanol by yeast, typically *S. cerevisiae*; 8) the ethanol is recovered by distillation (S. Lee & Shah, 2013).

The dry-milling process has the advantage of being less capital-intensive (R. J. Bothast & Schlicher, 2005), while the wet-milling process has the advantage of allowing for more flexibility in the product portfolio from the grain, for example, corn oils or corn meals can be extracted prior to fermentation in wet-milling operations (S. Lee & Shah, 2013). In 2005, 67% of fuel ethanol was produced by dry grind processes, while the remaining 33% was produced by wet grind processes (R. Bothast & Schlicher, 2005). In 2014, 90% of ethanol facilities were dry milling facilities (Alternative Fuels Data Center, 2013; Renewable Fuels Association, 2014b). It should be noted that these numbers cannot be directly compared, because the former presents the percentage of ethanol produced by dry milling facilities while the latter presents the percentage of facilities using the dry milling process. Both fermentation processes have similar ethanol yields; the industrial average ethanol yield in 2014 is 10.8 L/25.4 kg bushel of corn (Renewable Fuels Association, 2014b), which is near the theoretical maximum yield based on the starch content of the grain. It should be noted that wet grind corn facilities do not always produce bioethanol as a major product. They often sell the starch as unmodified cornstarch or convert the starch to syrups that are sold as food ingredients (Corn Refiners Association, 2005).

1.1.1.1 Co-products in first generation ethanol facilities

Co-products of bioethanol production play an important role in the profitability of the processes. In 2012 and 2013, the estimated value of co-products in the U.S. ethanol industry was $9.8 billion (Renewable Fuels Association, 2014a). Wet-grind corn facilities have a large array of co-products that include corn oil, feed products, starches, and sweeteners (Corn Refiners Association, 2005) and a number of "bioproducts" that include organic acids, amino acids, vitamins, and food gums (Corn Refiners Association, 2014). In comparison, there are two major co-products in dry-grind ethanol facilities: distillers grains, and corn distillers oil. Together, these co-products account for 27% of the gross revenue from a typical dry mill ethanol facility

(Renewable Fuels Association, 2014a). Because of the complexity and distinctiveness of each individual wet grind process, there are no industry average numbers for co-product value.

The product that remains in a dry grind ethanol facility after distillation is called wet distillers grains (WDGS) (S. Lee & Shah, 2013). This product can be dried to increase stability and transportability and it is then known as dried distillers grains (DDG) (S. Lee & Shah, 2013). The product is often mixed with the remains of the wet stillage to create dried distillers grains and solubles (DDGS) (S. Lee & Shah, 2013). In industrial settings, each bushel (25.4 kg) of corn yields between 7.7 and 8.2 kg of distillers grains (Renewable Fuels Association, 2014b). Industrial reports refer to the distillers grains as "enhanced" feed (Renewable Fuels Association, 2014a) because the conversion of the starch to ethanol results in the other nutrients in the feed, such as protein, to be concentrated through the process (the protein and fat content per kilogram of feed increases because the starch is removed). Distillers grains have been extensively studied and reviewed for use in the dairy (Schingoethe, Kalscheur, Hippen, & Garcia, 2009), beef (Klopfenstein, Erickson, & Bremer, 2008), swine (Stein & Shurson, 2009) industries, and studies are beginning to emerge for feed substitution in poultry (Swiatkiewicz & Koreleski, 2008) and aquaculture (Schaeffer, Brown, Rosentrater, & Muthukumarappan, 2010). Generally, these studies recommend blending distillers grains with conventional feed at a 5-30% distillers grains to conventional feed ratio, depending on the species and time in the feeding cycle, although distillers grains can be blended at up to 50% in some instances. In 2013, 48% of the distillers grains were consumed by beef cattle, 31% by dairy cattle, 12% by swine, 8% by poultry, and 1% by other species (Renewable Fuels Association, 2014a).

The second major co-product of dry grind facilities is distillers oil. Distillers oil contributed approximately $700 million to the gross earnings of the industry in 2013 (Renewable Fuels Association, 2014a). Distillers oils are extracted from the dried distillers grain using a solvent and can then be refined and used to produce biodiesel or food grade oil (Bruinsma & Endres, 2012; Bruinsma, Endres, & Furcich, 2013).

1.1.1.2 Criticisms of first generation ethanol production

First generation bioethanol mainly uses corn, or alternatively wheat, as a carbon source for fermentation (Evans, 2013; Renewable Fuels Association, 2014a). Because these are food

crops, the diversion of food crops and land for food production from food to first generation biofuels in North America met severe global criticism in 2008, particularly because global food prices had spiked during this time period (Baffes & Haniotis, 2010). Although the actual nominal impact of first generation biofuel production on food prices is contentious (Baffes & Haniotis, 2010), this backlash, along with the desire for less expensive, more readily available feedstocks promoted the development of second generation biofuels from non-food feedstocks (Antizar-Ladislao & Turrion-Gomez, 2008).

1.1.2 Second generation bioethanol

Second generation biofuels are produced from non-edible, lignocellulosic feedstocks such as wood, agricultural residue, and waste paper. Cellulosic biofuels, including ethanol, can be produced through thermochemical or biological pathways. Thermochemical pathways for the production of second generation ethanol typically proceeds through four general steps, although each process is unique: 1) conversion of biomass to syngas through steam gasification; 2) cleanup of syngas to remove methane, hydrocarbons, particulate, ammonia, halides, and tars; 3) alcohol synthesis in a catalytic fixed bed reactor; 4) and separation of alcohol using a molecular sieve followed by distillation (Dutta et al., 2012). This process typically produces a mixture of alcohols, including methanol, ethanol and higher molecular weight alcohols, which is why the separation step is required; sale of these co-products contributes to plant profitability (Dutta et al., 2012). A recent technoeconomic assessment indicates that production of bioethanol through thermochemical conversion will be industrially feasible only if improvements to catalyst technology are made (Dutta et al., 2012).

Biological conversion of lignocellulosic biomass gives a higher upper limit to bioethanol production compared with thermochemical conversion processes, although yields from both processes depend heavily on the feedstock and subprocessing steps, neither of which have been fully optimized in second generation biofuel production (Mabee & Saddler, 2010). Biological conversion generally proceeds through a 1) prehydrolysis of biomass through various treatments such as steam explosion, acid hydrolysis, solvent treatment, and/or ionic liquid pretreatment to fractionate the lignocellulose into cellulose, xylose, and lignin-rich fractions; 2) acid or enzymatic hydrolysis of the cellulose fraction to produce glucose; 3) fermentation of the glucose to produce ethanol; 4) separation of the ethanol (Lee & Shah, 2013).

1.1.3 Policy drivers for industrial bioethanol production

The Renewable Fuels Standard, part of the United States Energy Policy Act of 2005 and the Energy Independence and Security Act of 2007, as well as the Canadian Renewable Fuels Regulations (2010), have led to a substantial increase in production of bioethanol in North America. The Renewable Fuels Standard in the United States dictates the total volume of ethanol that may be produced through first and second generation technologies (Energy independence and security act, 2007); while the Canadian Renewable Fuels Regulations dictate blending requirements (Canadian environmental protection act: Renewable fuels regulations, 2010). In Canada, these standards were officially put in place to reduce greenhouse gas emissions (Canadian environmental protection act: Renewable fuels regulations, 2010), while in the United States the purpose of the act was to move towards increased energy security, reduce greenhouse gas emissions, and promote the use of renewable fuels (Energy independence and security act, 2007).

In the United States, the first and second generation bioethanol production capacities totaled 52.9 billion liters in 2014 (Renewable Fuels Association, 2014a), which is an increase from the 16.1 billion liters generated in 2006 (Renewable Fuels Association, 2006). Because of the Renewable Fuels Standard, combined bioethanol production from first and second generation feedstocks is expected to reach 136.3 billion liters by 2022 (Renewable Fuels Association, 2014a). The majority of growth in the industry is expected to be in second generation technologies because current first generation ethanol production has met the production quota established by the Renewable Fuels Standard (Renewable Fuels Association, 2014a).

In Canada, gasoline must be blended with a minimum average of 5% biologically-derived ethanol, while some provinces mandate up to 8.5% average blending (Evans, 2013). In order to meet these blending requirements, production capacity of first generation bioethanol has increased from 340 million liters in 2006 to 1.8 billion liters in 2014 (Evans, 2013). Second generation ethanol facilities add an additional 43 million liters of production capacity annually (Evans, 2013).

1.2 Microbial contamination in the bioethanol industry

There are two well-known microbial contaminants in bioethanol production: lactic acid bacteria (LAB) and the wild yeast *Dekkera bruxellensis*; LAB are the primary contaminants in the bioethanol industry, while wild yeasts are known to play a smaller role (Beckner, Ivey, & Phister, 2011). Contamination of industrial fermenters by *D. bruxellensis* has been reported in a number of studies (Abbott, Hynes, & Ingledew, 2005; Abbott & Ingledew, 2005; Elsztein, Scavuzzi de Menezes, & de Morais, 2008; Liberal et al., 2007; Muthaiyan & Ricke, 2010; Tavares, 1995). Studies of co-cultures designed to mimic *D. bruxellensis* contamination in *S. cerevisiae* fermentation suggest that *D. bruxellensis* lowers the overall fermentation efficiency (Meneghin, Guarnieri Bassi, Codato, Reis, & Ceccato-Antonini, 2013). Although the mechanism of the lowered fermentation efficiency is not completely understood, it has been shown that *D. bruxellensis* diverts much of the carbon to biomass rather than ethanol production (Pereira, Lucatti, Basso, & de Morais, 2014). Because *D. bruxellensis* can metabolize ethanol and is highly-ethanol tolerant (Dias, Pereira-da-Silva, Tavares, Malfeito-Ferreira, & Loureiro, 2003), Beckner *et al.* (2011) hypothesize that *D. bruxellensis* may out-compete *S. cerevisiae* during later stages of the fermentation and metabolize ethanol, reducing the overall yield. Since most fungicides active against wild yeasts are also active against *S. cerevisiae* it is difficult to control fungal contamination in a fungal fermentation. Thus, the recommended response to wild yeast contamination is a cleaning and changeover of the entire yeast population of the bioreactor (Liberal et al., 2007).

Contamination of the bioreactor by LAB is a chronic issue in the bioethanol industry (Beckner et al., 2011). Due to their tolerance for low oxygen, low pH, and high ethanol concentrations, LAB are well adapted to thrive during ethanol fermentations (Beckner et al., 2011). LAB have been known to contaminate both wet and dry grind ethanol facilities. Skinner and Leathers (2004) found final cell counts of 10^6 colony forming units/mL (CFU/mL) in a wet grind facility and up to 10^8 CFU/mL of contaminating bacteria in two dry grind facilities. The bacteria in these facilities were predominantly *Lactobacillus* sp (Skinner & Leathers, 2004). LAB have also been reported to reduce yields in cellulosic ethanol fermentations (Isci et al., 2009). LAB infections can occur within the grain mash, but can also form biofilms on production

equipment, increasing the complexity of the treatment and cleaning process (Rich, Leathers, Nunnally, & Bischoff, 2011).

The production yield reduction from an individual LAB contamination event has been modeled to be as high as 27% (Bischoff, Liu, Leathers, Worthington, & Rich, 2009). This is a substantial loss on its own; however, in many industrial situations, the best option to remove contamination and prevent further contamination is to shut down and clean all of the production equipment, accumulating substantial losses in production time during the shutdown (Ebert, 2007). Estimates of these losses to the bioethanol industry as a whole are discussed in section 1.2.1.

There are two major mechanisms that have been thought to contribute to ethanol production losses due to contamination by LAB: competition with the fermentation yeast for nutrients including glucose (K. Thomas, Hynes, & Ingledew, 2001), and the production of organic acids (D. Bayrock & Ingledew, 2001; D. P. Bayrock & Ingledew, 2004; Graves, Narendranath, Dawson, & Power, 2006; N. V. Narendranath, Hynes, Thomas, & Ingledew, 1997; N. V. Narendranath & Power, 2005; K. Thomas, Hynes, & Ingledew, 2002), which are toxic to yeast.

In addition to their individual contributions to contamination events, *D. bruxellensis* and species of LAB are thought to form stable consortia in long-term fermentations where the yeast is reused (Passoth, Blomqvist, & Schnurer, 2007). In the study where the consortia were first recognized, they had overtaken the *S. cerevisiae* population, but still produced an acceptable yield of ethanol (Passoth et al., 2007). In a further study of the interaction of the LAB and *D. bruxellis*, Tiukova *et al.* (2014) found that *D. bruxellensis* and LAB form flocs, especially in the presence of high ethanol concentrations, and hypothesize that the flocculation activity protects the inner cells from high ethanol concentrations. Thus, *D. bruxellenisis* may play a role in aiding the survival of contaminating LAB in bioethanol fermentations; this interaction is a potential area of interest for preventing LAB contamination.

1.2.1 Losses from bacterial contamination

Although there are no reports on the actual extent of production losses due to contamination, experts conservatively estimate the losses at 1% to 4% (Retka Schill, 2013). Based on a 52.9 billion liter per year production capacity in the U.S. (Renewable Fuels Association, 2014a), 1 to 4 % losses in production translate to 529 million to 2.1 billion liters in production losses. In August 2014, the average price of ethanol was $0.52/L (USDA, 2014), so this 529 million to 2.1 billion liter annual production loss translates to $275 million to $1.1 billion annual losses for the industry.

1.3 Control of bacterial contamination using conventional antibiotics

Penicillin and virginiamycin are the most widely used antibiotics in the bioethanol industry (Muthaiyan, Limayem, & Ricke, 2011). Muthaiyan *et al.* (2011) estimate that an ethanol plant that produces 473 million liters per year spends approximately $40 000 USD on antibiotics annually. When extrapolated to the entire bioethanol industry, the total expenditure on antibiotics is approximately $4.4 million USD.

Bayrock *et al.* (2003) studied both the constant and pulsed addition of penicillin G to continuous ethanol fermentations intentionally infected with *Lactobacillus paracasei* and found that both pulsed and continuous additions of the antibiotic controlled the infection and eliminated the reduction in ethanol yield caused by the infection. Penicillin G is a member of the β-lactam group of antibiotics that work by inhibiting the transpeptidation step of peptidoglycan synthesis. These antibiotics are generally considered broad-spectrum and are widely used in clinical settings (Nikolaidis, Favini-Stabile, & Dessen, 2014). There are four common resistance mechanisms to β-lactam antibiotics: 1) reduced membrane permeability or increased drug efflux via efflux pumps; 2) expression of penicillin target proteins that have lower affinity for the drug; 3) using an alternate cell wall cross-linking mechanism; and 4) expression of β-lactamases that degrade the antibiotic (Nikolaidis et al., 2014).

In a study where virginiamycin (in the form of Lactrol®, a commercial preparation used later in this study) was added to a wheat mash-based ethanol fermentation, it also reduced LAB counts and relieved the effect of the LAB on the reduced ethanol yield (Hynes, Kjarsgaard, Thomas, & Ingledew, 1997). Virginiamycin is a member of the streptogramin family of

antibiotics that inhibit peptide synthesis by binding to ribosomes and preventing interactions between ribosomes and protein elongation factors (Chinali, Moureau, & Cocito, 1981). In LAB, the most common resistance mechanisms to streptogramins are modification of the ribosome to prevent drug binding, drug efflux via efflux pumps, and enzymatic inactivation of the antibiotic (Thumu & Halami, 2012).

Antimicrobial resistant strains of LAB and LAB containing genes for antimicrobial resistance (acquired through lateral transfer) isolated from ethanol facilities were first reported in trade publications (Lushia & Heist, 2005). In 2007, a study of a dry-grind ethanol facility that periodically dosed with virginiamycin found isolates that had a decreased susceptibility to virginiamycin compared with isolates from facilities that did not dose with virginiamycin (Bischoff, Skinner-Nemec, & Leathers, 2007). Sixteen of 42 isolates from the facility that dosed with virginiamycin possessed the vatE gene, a gene that encodes a streptogramin acetyltransferase, while none of the isolates from the virginiamycin-free facility possessed the gene (Bischoff et al., 2007). A recent study of LAB isolates from eight different dry-grind ethanol facilities found broad resistance to erythromycin, penicillin, and virginiamycin and that 23 of the 32 isolates possessed one or more antimicrobial resistance genes (Murphree, Heist, & Moe, 2014).

1.3.1.1 Antibiotic resistance as a public health concern

Other than the cost of antibiotics, the major opposition (discussed further in section 1.3.2) to the use of conventional antibiotics in ethanol fermentations is that the use of antibiotics will encourage the proliferation of antibiotic-resistance in microbes. The Word Health Organization considers antimicrobial resistance a serious threat to public health and recommends regulating the use of antibiotics in animals (World Health Organization, 2014). The majority of this concern has stemmed from the concern that LAB will become a reservoir for antimicrobial resistance genes that could then be transferred to pathogenic bacteria through lateral gene transfer (Devirgiliis, Zinno, & Perozzi, 2013).

1.3.1.2 Antibiotic residues and resistance in dried distillers grain

The use of antibiotics to control LAB in bioethanol production has become a contentious issue because of the potential for the presence of antibiotics in the distillers grains, which are

marketed as animal feed. There is one case described in literature where cattle fed DDGS unknowingly contaminated with a number of macrolide antibiotics were also fed the antibiotic monensin (Basaraba, Oehme, Vorhies, & Stokka, 1999). The combination of the antibiotic residues and monensin caused the death of 562 cattle (Basaraba *et al*., 1999).

In a review of the analysis of distillers grain microflora by Pedersen *et al.* (2004), it was suggested that even though the DG microflora is killed during distillation, it is reinoculated with the LAB from fermentation before leaving the ethanol facility. Muthaiyan *et al*. (2011) suggest that if these LAB harbour antimicrobial resistance, the antimicrobial resistance would be carried from the facility in the LAB. If antimicrobial resistance is carried from the facility in the distillers grains used as animal feed, this may encourage growth of resistance microbes in animals.

A recent study analyzed wet distillers grains and dried distillers grains from 43 ethanol facilities in the U.S. for the presence of five antibiotics using liquid chromatography, mass spectrometry, and biological activity assays (Paulus Compart et al., 2013). 13% of the distillers grains in the study had low, but detectable, levels of antibiotics and one sample had biological activity against *E. coli* (Paulus Compart et al., 2013). The authors concluded that the antibiotics were being degraded in the high temperatures present in the distillation and drying processing stages and that the low levels of antibiotics detected were unlikely to cause an increase in the levels of antibiotic resistant bacteria in livestock (Paulus Compart et al., 2013).

Another recent study evaluated the fecal microflora from cattle fed DG from ethanol fermentations that were either not dosed with virginiamycin, dosed with 2 or 20 mg/kg virginiamycin, or spiked with virginiamycin post-fermentation (Edrington, Bischoff, Loneragan, & Nisbet, 2014). There was very little difference in the antimicrobial resistance and the presence of the antimicrobial resistance gene *ermB* in the microflora between the dosed fermentations and the control (Edrington, Bischoff, Loneragan, & Nisbet, 2014). This study also concluded that feeding DDG from fermentations dosed with virginiamycin has a very low risk of impacting the prevalence of antimicrobial resistant strains (Edrington et al., 2014).

Although these studies reveal that the effects of antibiotic use in bioethanol facilities on the prevalence of antimicrobial resistant strains is controversial, the use of antibiotics may soon

become either highly regulated or prohibited by regulatory agencies. The U.S. Food and Drug Administration (USFDA) Food Safety Modernization Act (FSMA) was signed in 2011 and requires all producers of animal feed to follow current Good Manufacturing Practices, hazard analysis, and risk-based preventative control requirements (U.S. Food and Drug Administration, 2011). The USFDA has not yet made a final ruling on how the FSMA affects ethanol producers, how ethanol producers will be regulated, and if antibiotic use will be regulated (U.S. Food and Drug Administration, 2014)

1.3.2 Alternative control measures

1.3.2.1 Current industry practices

In addition to the use of antibiotics, several alternate control measures are already in use in industry. Acid washing of the yeast, especially prior to re-pitching, is common in Brazilian and Southeast Asian bioethanol facilities (Muthaiyan et al., 2011). Acid washing of yeast with phosphoric acid (pH 2.1) or sulfuric acid (pH 2.0) has been prevalent in the brewing industry for many years and reportedly controls contaminating bacteria without having a serious impact on the metabolism, viability, and ethanol production of the yeast if the process is performed correctly (the acid is chilled, the mixture remains below 5°C during the wash, and the yeast is pitched immediately following the acid wash) (Simpson & Hammond, 1989). However, in high-gravity fermentations, acid washing can hinder sugar metabolism early in the fermentation (Cunningham & Stewart, 1998). Cunningham and Steward (1998) do not speculate as to why acid washing can hinder sugar metabolism in high gravity fermentations, but it may be caused by the osmotic pressure of the high gravity (high sugar) fermentations on acid-damaged cells that prevents the cells from recovering from the acid wash.

Resonant Biosciences LLC markets a chlorine dioxide and hydrogen peroxide system to ethanol producers (Kram, 2008). Hydrogen peroxide is specifically toxic to LAB because LAB does not possess the catalase enzyme that degrades hydrogen peroxide (Condon, 1987). The use of hydrogen peroxide is also supported by literature: 1mM to 10mM concentrations of hydrogen peroxide were successfully used to selectively control *Lactobaccillus fermentum* contamination in a cell-recycled continuous ethanol fermentation process (Chang, Kim, & Shin, 1997). One limitation to the use of hydrogen peroxide is the expense of the delivery and storage system; to

mitigate this cost, researchers have experimented with the use of urea – hydrogen peroxide (UHP), a stable solid that releases urea and hydrogen peroxide when dissolved in water (N. Narendranath, Thomas, & Ingledew, 2000). Narendranath *et al.*(2000) found that 30 mM UHP addition mitigated the effect of five LAB species on ethanol production in a normal gravity wheat mash fermentation, and also suggest that the urea acts as a nitrogen source to improve the performance of the yeast. Although this system is effective, there are always costs associated with using additional chemicals during fermentation and there is potential for microorganisms to develop resistance to chemical measures.

Hop compounds, extracted from the flowers of the hop plant *Humulus lupulus*, are also being marketed to control LAB contamination in ethanol fermentations (BetaTec hop products, 2013). Hop compounds inhibit bacterial growth by embedding in the cell membrane and acting as ionophores, dissipating the proton motive force and causing leakage of the cell membrane (Simpson, 1993). Hops are traditionally added as a bittering agent and preservative to beer and have been studied for use in bioethanol production (Ruckle, 2005; Ruckle & Senn, 2006). Ruckle and Senn (2006) found that hop compounds used at a concentration of 90 to 160 ppm were as effective at controlling LAB contamination in a wheat mash fermentation as those containing 0.5 ppm virginiamycin or 0.25 ppm penicillin G. Although hops compounds are a promising additive to bioethanol fermentations to control LAB contamination, hop-resistance is a well-documented problem in the brewing industry (Behr, Gaenzle, & Vogel, 2006; Suzuki, Iijima, Sakamoto, Sami, & Yamashita, 2006). Hop resistance mechanisms include the presence of *horA*, a gene encoding an efflux pump (Sakamoto, Margolles, van Veen, & Konings, 2001) and alteration of cell wall composition (Behr et al., 2006).

1.3.2.2 Control measures in development

Several additional microbial control measures have been studied that have not yet been applied industrially. Broda and Grajek (2009) investigated the use of ammonia disinfection of corn prior to fermentation. The grain was treated with (0.5, 1.0, 1.5 % w/v) ammonia for up to two weeks and LAB counts were performed; after two weeks the LAB count was reduced from 4.56 log CFU/g to undetectable levels (Broda & Grajek, 2009). This reduction in LAB counts corresponded with a 2% (v/v) increase in ethanol production from the grain (Broda & Grajek, 2009). Muthaiyan *et al.* (2011) point out that although this process needs development before

application, it has the benefit of offering a nitrogen source (ammonia) for the yeast. Although this process may have potential, it would require the addition of grain holding tanks to ethanol facilities in order to be practical for use, requiring a large capital investment for the facilities.

Chitosan has also been investigated as an additive to selectively prevent the growth of LAB in ethanol fermentations (Gil, del Monaco, Cerrutti, & Galvagno, 2004). Chitosan is a linear polysaccharide derived from chitin, which is present in shells of crustaceans. Chitosan's antimicrobial mechanisms are poorly understood at the molecular level, but it is generally believed to interact electrostatically with membrane lipids, causing membrane leakage and damage (Kong, Chen, Xing, & Park, 2010). Gil *et al.* (2004) co-fermented yeast and LAB in malt extract containing no chitosan or 0.1 g/L chitosan and found that 0.1 g/L chitosan inhibited bacterial growth, while it did not affect the yeast growth or fermentation yield. This is a promising study, but further work needs to be done to study the performance in high gravity grain mash systems in order to mimic industrial conditions.

The use of bacteriophage has also been explored for use in bioethanol facilities, (Silva & Sauvageau, 2014) and for use in beer to control beer spoilage (Deasy, Mahony, Neve, Heller, & van Sinderen, 2011). Deasy *et al.* (2011) isolated a phage capable of controlling contamination during beer production through infection of a *Lactobacillus brevis* that causes cell lysis. The phage was also effective against three other beer spoilage strains of *La. brevis* of the panel of 22 strains tested in the study (Deasy et al., 2011). Silva & Sauvageau (2014) used a cocktail of two phages to control the growth of *La. plantarum* in M9 media and restore ethanol production that was inhibited by the presence of the bacteria. Silva & Sauvageau (2014) list several considerations for the use phage as a control mechanism: the relatively narrow spectrum of activity, the suitability of industrial fermentation conditions for phage usage, the presence of phage in the distillers grains, all of which would have to be studied prior to the commercialization of this technology. Once these are studied, low cost of phage and the low potential for resistance make phage a promising treatment for LAB contamination (Silva & Sauvageau, 2014).

Although bacteriophage is relatively specific in its spectrum of activity, bacteriophage endolysin can be active across several species (Khatibi, Roach, Donovan, Hughes, & Bischoff, 2014). Khatibi *et al.* (2014) expressed a phage endolysin active against several strains of LAB in

S. cerevisiae. When applied externally, phage endolysins degrade the peptidoglycan cell walls of Gram positive bacteria, causing osmolysis (Borysowski, Weber-Dabrowska, & Gorski, 2006). They found modest reductions in LAB counts (ranging from 0.9 log CFU/mL to 3.3 log CFU/mL, depending if the fermentations were treated with yeast cells or lysed endolysin-expressing yeast cells, respectively) when the endolysin-producing *S. cerevisiae* strains were used in corn mash fermentations, compared with strains that were not expressing and secreting the endolysin (Khatibi et al., 2014). While these results are indeed modest, further development of modified yeast strains could have a substantial effect on the bioethanol industry.

The use of bacteriocins to control LAB in bioethanol fermentations has also been studied. Bacteriocins, explained in more depth in section 1.4, are peptides or small proteins secreted by lactic acid bacteria that kill closely related bacteria. Peng *et al.*(2012) used the bacteriocin nisin (I5 IU/mL) to control LAB in ethanol fermentations. This nisin treatment decreased LAB counts, increased ethanol production, and decreased lactic acid production in the treated fermentations compared to the control where no nisin was added (Peng et al., 2012). In the conclusion of the study, Peng *et al.*(2012) allude to the potential for the development of a strain of *S. cerevisiae* capable of secreting nisin.

Although no strain of *S. cerevisiae* capable of secreting nisin has been developed, work has been done to engineer strains of *S. cerevisiae* that secrete bacteriocins (Basanta et al., 2009; Schoeman, Vivier, Du Toit, Dicks, & Pretorius, 1999; Van Reenen, Chikindas, Van Zyl, & Dicks, 2003). None of these groups were successful in developing strains that secreted industrially-relevant concentrations of bacteriocins. This work is summarized in detail in section 2.1.

1.3.3 Combining control measures – using the hurdle concept in the ethanol industry

The use of hurdle technology, where several antimicrobial "hurdles," each used at a relatively mild concentration, is used routinely in the food industry to combat food spoilage (Leistner & Gorris, 1995). These "hurdles" can include factors like water activity, pH, pressure treatment, temperature treatment, and salt concentration applied simultaneously or in sequence. Hurdle technology could potentially be applied to the bioethanol industry, where several practices, for example, a combination of hydrogen peroxide treatment, hop compounds addition,

and a bacteriocin-secreting yeast could be combined to prevent growth of LAB. Combinations of inhibitors can produce additive, synergistic, or antagonistic effects when combined in treatment. Further studies are required to ensure that when antimicrobials are combined, the effect is additive or synergistic, rather than antagonistic.

1.4 Bacteriocins

Bacteriocins are typically defined as ribosomally-synthesized peptides that are produced by bacteria and inhibit the growth of other closely-related bacteria. In a recent review, Snyder and Worobo (2014) point out that both the "closely-related" and "peptide" parts of this definition can be debated; some bacteriocins are known to have a broad spectrum of activity, and some can be considered small proteins rather than peptides. Klaenhammer (1993) divided bacteriocins into four distinct classes; several modifications to this classification system have been made since, most recently by Heng and Tagg (2006). There are currently four generally-accepted classifications for bacteriocins: Class I, lantibiotics; Class II, non-lantibiotic, unmodified peptides; Class III, large, heat stable; Class IV, cyclic (Heng & Tagg, 2006).

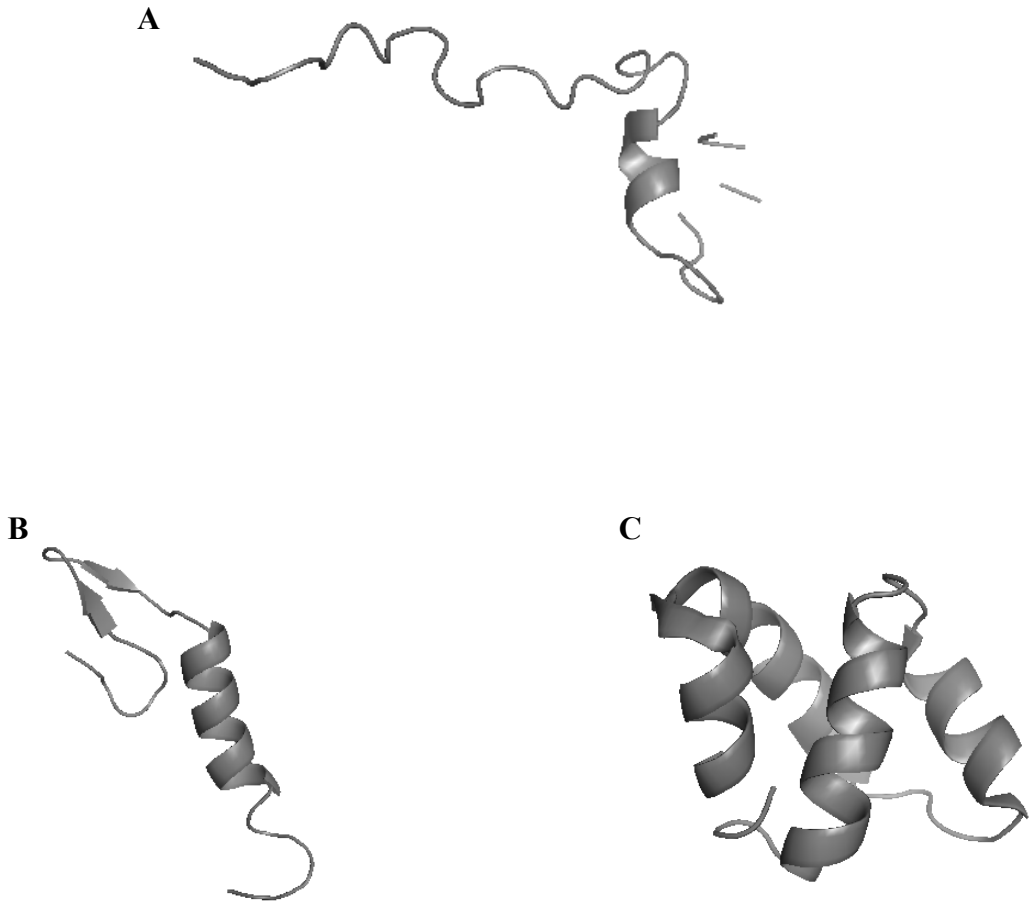


Figure 1-1. Resolved three dimensional structures of three bacteriocins used in this study. Nisin, a Class I bacteriocin (A) (Hsu et al., 2004), Leucocin A, a Class IIa bacteriocin (B) (Wang et al., 1999), and Carnocyclin A (C), a Class IV bacteriocin (Martin-Visscher et al., 2009)-v. Structures generated using Pymol (Shrodinger, New York, USA)

1.4.1 Class I bacteriocins

Class I bacteriocins, more typically referred to as lantibiotics, are small peptides that are post-translationally modified to contain several unusual amino acids: lanthionine and 3-methyllanthionine, which form ring structures within the molecules, and dehydroalanine, dehyrobutyrine, D-alanine, formyl methionine, and residues containing 2-oxobutyryl moieties (Nishie, Nagao, & Sonomoto, 2012). These post-translational modifications contribute to the relative stability of the molecules to a wide pH and temperature ranges, oxidation, and proteolysis (Bierbaum et al., 1996; Sahl, Jack, & Bierbaum, 1995).

Nisin (Figure 1-1A), produced by *Lactococcus lactis,* is arguably the most commonly known bacteriocin and has a wide range of commercial applications. Nisin activity was first discovered in 1928 (Rogers & Whittier), used as a food preservative in England in 1953, and has been approved for use in 48 countries since (Ross, Morgan, & Hill, 2002). Nisaplin® and Novasin™, are two nisin-containing products currently marketed to food companies as "natural antimicrobials" against Gram-positive bacteria, for use in dairy products, dressings, sauces, meat products, liquid eggs, canned foods, and crumpets (Gillco Ingredients, 2014). Nisin is used later in this study.

Nisin's mechanism of action occurs through two steps: 1) receptor binding, and 2) pore formation. The nisin receptor is the cell-wall precursor lipid II (Brotz et al., 1998). Following receptor binding, pore formation in the target cell membrane occurs. The mechanism of pore formation is still disputed, but most recent studies indicate that nisin binding promotes a "wedge" mechanism that causes the membrane phospholipids to reorient, forming a short-lived pore lined by phospholipid head groups (Figure 1-2) (Asaduzzaman & Sonomoto, 2009; Moll, Konings, & Driessen, 1999). Several nisin molecules are likely involved in the formation of this transient pore (Moll et al., 1999).

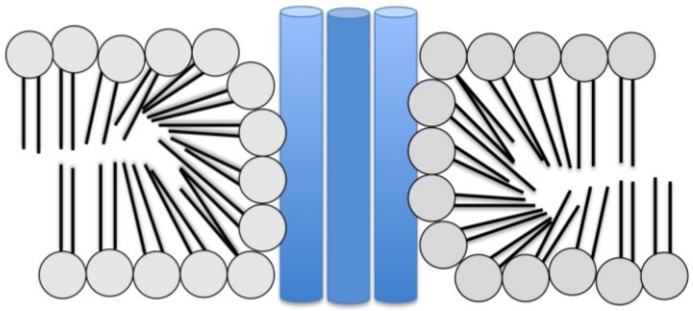


Figure 1-2 Wedge model of bacteriocin pore formation. Several helices (represented in blue above) are involved in the formation of the transient pore.

1.4.2 Class II bacteriocins

Class II bacteriocins are peptide bacteriocins classified as non-lantibiotic bacteriocins that have anti-*Listeria* activity, although neither Class III and Class IV bacteriocins contain lanthionine residues (Snyder & Worobo, 2014). Class II has become a "catch-all" subclass for bacteriocins and the class is further subdivided into several subclasses; the number of subclasses has been somewhat controversial because bacteriocins are continually being discovered; however, most recent literature divides Class II into three classes: Class IIa, pediocin-like bacteriocins; Class IIb, miscellaneous bacteriocins; and Class IIc, multicomponent bacteriocins (Snyder & Worobo, 2014).

Class IIa, also known as pediocin-like bacteriocins, are used later in this study. Bacteriocins in this subclass range from 37-48 amino acids in length and contain a conserved, positively charged N-terminal region containing the consensus sequence YGNGVXCX(K/N)XXCXV(N/D)(W/K/R)X(G/A/S)(A/N), although there are some exceptions to this consensus sequence among bacteriocins classified as Class IIa. (Ennahar, Sashihara, Sonomoto, & Ishizaki, 2000). The C-terminus of Class IIa bacteriocins contains one or two hydrophobic or amphipathic α-helices (Ennahar et al., 2000). Leucocin A (Figure 1-1B), a member of this class, is used later in this study.

Like Class I bacteriocins, Class IIa bacteriocins act through two sequential processes: first by receptor recognition, then by forming pores in the cell membrane. The receptor for Class IIa bacteriocins is an extracellular loop of the mannose phosophotransferase system (Kjos, Salehian, Nes, & Diep, 2010). Early studies of the structure-function relationship of Class IIa bacteriocins established that the amphipathic or hydrophobic α-helix is involved in pore formation (Chen, Ludescher, & Montville, 1997; Fimland, Jack, Jung, Nes, & Nissen-Meyer, 1998; Fleury et al., 1996b; Kaiser & Montville, 1996). The specific molecular mechanism of this pore formation is not clear, although it is thought to involve aggregation of several bacteriocin molecules to create water-filled pores (BhugalooVial et al., 1996; Chen et al., 1997; Fleury et al., 1996a), possibly through a barrel-stave mechanism (Ennahar et al., 2000).

Although Class IIa bacteriocins are generally referred to as "non-modified" bacteriocins, they do undergo one important post-translational modification: the cleavage of a leader peptide, usually ending in conserved GG residues, from the N-terminus of the translated peptide (van Belkum, Worobo, & Stiles, 1997). This N-terminal processing is thought to occur during protein export; the N-terminus may act as a type of signal sequence or may be involved in rendering the bacteriocin inactive while it is within the host cell (Havarstein, Diep, & Nes, 1995). In addition to the export/processing machinery, Class IIa bacteriocins are co-expressed with a number of additional proteins involved in synthesis, processing/export, immunity, and regulation (Ennahar et al., 2000).

The boundaries of the Class IIb and IIc bacteriocins have recently changed. Class IIb includes "miscellaneous" bacteriocins that are unmodified and leaderless but do not fit into the Class IIa category, while Class IIc includes multi-subunit bacteriocins (Heng & Tagg, 2006). These bacteriocins were not used in this study due to the complexity of coordinating the secretion of multiple peptides.

1.4.3 Class III bacteriocins

Class III bacteriocins are large, globular, and generally heat-labile molecules that can be sub-classified into two subclasses: bacteriolytic and non-bacteriolytic (Heng & Tagg, 2006; Snyder & Worobo, 2014). Due to their heat lability, class III bacteriocins are not used in this study. Heat-lability makes these bacteriocins a poor candidate for industrial use.

1.4.4. Class IV bacteriocins

Class IV bacteriocins are the most recent addition to the bacteriocin classification system (Snyder & Worobo, 2014). These bacteriocins are small (carnocyclin A (Figure 1-1C), used in this study, is 6.4 kDa), head-to-tail cyclic peptides and are sometimes referred to as circular bacteriocins (Martin-Visscher, van Belkum, & Vederas, 2011). Due to their circular structure, bacteriocins in the class maintain activity when exposed to high temperatures, chemical treatments, and proteases (Martin-Visscher et al., 2011). The two elucidated three-dimensional structures of bacteriocins in this class, enterocin AS-48 and carnocyclin A, indicates that the two peptides are very similar in three-dimensional structure, despite large differences in their primary sequences; the proteins consists of four or five α-helices surrounding a hydrophobic core (Martin-Visscher et al., 2011). Little is known about the mechanism of cyclization, although there are likely a number of accessory proteins involved in the cyclization process (Martin-Visscher, Yoganathan, Sit, Lohans, & Vederas, 2011).

Because most circular bacteriocins are relatively new discoveries, the mechanism of action is poorly understood, and it is not known if there is a receptor-binding step. It is also unclear if the mechanism of action is conserved within this class: the circular bacteriocin carnocyclin binds to the membrane in a voltage-dependent manner and forms anion-selective channels (Gong, Martin-Visscher, Nahirney, Vederas, & Duszyk, 2009), while enterocin AS-48 forms non-selective pores (Galvez, Lopez, Abriouel, Valdivia, & Omar, 2008). Recent studies have indicted that conformational changes in AS-48 are induced upon membrane binding, causing pore formation through a "leaky slit" mechanism that is similar to the wedge mechanism proposed for nisin (Cruz, Ramos, Melo, & Martinez-Salazar, 2013).

1.5 Industrial microorganisms

1.5.1 Saccharomyces cerevisiae

In addition to bioethanol production, *S. cerevisiae* has been traditionally used industrially to produce beer, bread, and wine (J. Nielsen & Jewett, 2008). Due to the yeast's Generally Regarded As Safe (GRAS) status, pH tolerance, relative tolerance to fermentation inhibitors

(Borodina & Nielsen, 2014), as well as the fact that the organism is very well characterized genetically (J. Nielsen & Jewett, 2008), *S. cerevisiae* has become an organism of choice for systems biologists and metabolic engineers. In industry, very recent advances in metabolic engineering of *S. cerevisiae* have led to commercialization of several platform chemicals from biomass, including lactic acid (produced by Natureworks LLC), farnesene (Amyris), resveratrol (Evolva), ethylene (Braskem), succinic acid (Reverdia), and isobutanol (GEVO) (Borodina & Nielsen, 2014). The array of bioproducts produced in *S. cerevisiae* by academics at a laboratory scale is ever-expanding and most recently has included itaconic acid, a petrochemical replacement for bioplastic production (Blazeck et al., 2014), triacetic acid lactone, a sorbic acid precursor, (Cardenas & Da Silva, 2014), and chanoclavine-I, a biologically-active ergot alkaloid intermediate compound (C. A. F. Nielsen et al., 2014).

In addition to platform chemicals, *S. cerevisiae* has also been used to produce recombinant proteins as bioproducts including human insulin, hepatitis vaccines, and human papillomavirus vaccines (Hou, Tyo, Liu, Petranovic, & Nielsen, 2012). However, Roslyn Bill recently noted in a review that *S. cerevisiae* is "surprisingly underused" as a recombinant production host, especially when compared with *E. coli* (Bill, 2014). One of the challenges faced when using *S. cerevisiae* as a recombinant production and secretion host is: that there is not a complete understanding of the secretory pathway, including models for each process within the secretory pathway (Hou et al., 2012). Despite these challenges, the use of *S. cerevisiae* to produce recombinant proteins as a bioproduct is a desirable outcome, especially given the growing use of *S. cerevisiae* for other biotechnical applications, as outlined above.

1.5.2 Escherichia coli

E. coli is used to produce up to 30% of commercial recombinant proteins used in biopharmaceutical applications (Ferrer-Miralles, Domingo-Espin, Corchero, Vazquez, & Villaverde, 2009) and up to 70% of laboratory recombinant proteins (Bill, 2014). *E. coli* is a popular choice for recombinant protein production because of its well-characterized genetics, high yields, general simplicity of scale-up, and low media costs (Huang, Lin, & Yang, 2012). *E. coli* is generally not used in bioethanol production, but because it is commonly used for recombinant protein production, it is used in this study in an attempt to produce circular recombinant proteins.

1.6 Research goals

As stated in section 1.2.1, microbial contamination is a major source of inefficiency in the bioethanol industry. Conventional treatments for contamination are expensive, and potential regulatory changes may soon prevent the use of the most common treatment, antibiotic addition. Because of the expensive nature and the potential regulation of the industry as well as the growing importance of the ethanol industry, it is increasingly important to improve the antimicrobial regimens of bioethanol facilities.

One potential improvement to microbial treatment regimens in industrial bioethanol production is to use microbial inhibitors in combination. When used in tandem, some combinations of microbial inhibitors will have additive or synergistic properties, which leads to a lower effective dose and cost savings. However, combinations of microbial inhibitors may also have antagonistic activity that will have the opposite effect. Thus, it is important to study these interactions in order to optimize the use of multiple inhibitors during bioethanol production.

A second improvement to treating microbial infections in bioethanol production could be to develop a strain of *S. cerevisiae* that secretes bacteriocins into the fermentation media. Bacteriocins specifically target the lactic acid bacteria involved in microbial contamination. A strain that effectively secretes bacteriocins could improve the efficiency of contaminated fermentations, as well as prevent contamination events from occurring. A bacteriocin-secreting yeast strain could also be used in tandem with other microbial inhibitors, depending on the interactions between bacteriocins and the other microbial inhibitors.

As stated in section 1.4.4, circular bacteriocins are highly stable and this property makes them a potentially valuable bioproduct for use in bioethanol fermentations, but also for broader applications. Because the regular native pathways for circular bacteriocin production are poorly understood, heterologous expression of the bacteriocin may be a viable route to commercial production. Expression of the bacteriocin could be performed first in *E. coli* and then potentially in a different expression host or used in a yeast system to protect bioethanol or other fermentation applications.

Inexpensive DNA sequencing and DNA synthesis technologies have led to rapid advancements in metabolic engineering of yeast. This has led to a large array in fermentation

products and more efficient fermentations. Alternative cloning strategies for *S. cerevisiae* are included in these advancements and it may be possible to further support these rapid advancements with the development of additional strategies.

The objectives of this study are as follows:

1. Develop microbial strains for heterologous bacteriocin production:
    a. *S. cerevisiae* that secretes bacteriocins as a bioprotective strategy for bioethanol fermentation
    b. Develop a strain of *E. coli* that produces the circular bacteriocin carnocyclin
2. Evaluate industrially-available microbial inhibitors used during bioethanol production for additive and synergistic properties
3. Develop alternate cloning strategies for genetic manipulations in *S. cerevisiae* that would expedite further creation of yeast strains described in 1a.

Chapter 2: Secretion of Leucocin A from **Saccharomyces cerevisiae**

2.1 Introduction

Bioreactor contamination by lactic acid bacteria (LAB) is a serious problem in the bioethanol industry; facilities can experience cases of contamination where counts of LAB can reach 10^8 CFU/mL (Skinner & Leathers, 2004). Contamination by LAB can significantly decrease the ethanol yield; in model cases the ethanol yield can be reduced by up to 27% (Bischoff et al., 2009). The decrease in yield in contaminated fermentations is due to two effects of the LAB on the fermentation: 1. LAB compete with the fermentation yeast for nutrients, and 2. LAB produces organic acids, mainly lactic acid, which are toxic to the yeast in their undissociated form (Bayrock & Ingledew, 2004).

The addition of antibiotics such as penicillin G and viginiamycin to the bioreactor in order to control the LAB growth has been studied (Bayrock et al., 2003; Hynes et al., 1997) and the United States Federal Department of Agriculture (USDA) believes this to be a highly adopted practice in the ethanol industry (McChesney, 2009). Although the use of these antibiotics can successfully control LAB growth and restore ethanol yields, there is opposition to the use of these antibiotics in an industrial setting. One criticism of the use of antibiotics has been that residual antibiotics from the fermentation may remain in the distillers grains following fermentation. Distillers grains are fed to livestock and there is currently opposition to feeding livestock unnecessary antibiotics that could breed antibiotic-resistant strains. A study on the presence of residual antibiotics in distillers grains from 43 ethanol facilities was completed in 2013: 13% of dried distillers grains samples contained detectable amounts of antibiotics and one of these samples had biological activity (Paulus Compart et al., 2013). While Paulus Compart *et al.* (2013) evaluate these levels as a low risk to causing human health repercussions through the development of antibiotic resistant strains, antibiotic addition may still contribute to the rise in transferable antibiotic resistance genes (Sunde, Fossum, Solberg, & Sorum, 1998).

Several alternatives to the use of conventional antibiotics exist and are discussed in depth in Chapter 4. One alternative that has been explored but is not yet commercially viable is the development of strains of *Saccharomyces cerevisiae* that secrete bacteriocins. Bacteriocins are a class of antimicrobial proteins and peptides secreted by LAB that generally kill related LAB

(Klaenhammer, 1993). There are four classes of bacteriocins: Class I, which includes nisin and other lantibiotics; Class II, small, heat-stable peptides that do not get post-translationally modified; Class III, large, heat-sensitive proteins ; and Class IV; cyclic bacteriocins (Heng & Tagg, 2006). Nisin, a Class I bacteriocin, is the most commonly used bacteriocin for industrial applications (Nishie et al., 2012); however, nisin and other lantibiotics undergo a large degree of post-translational modification prior to maturation (Klaenhammer, 1993). The need for post-translational modification makes nisin and other Class I bacteriocins undesirable for secretion in yeast because post-translational modifications require additional accessory proteins. Expression of several accessory proteins in a coordinated fashion in order to produce the modified proteins would dramatically increase the complexity of expressing the protein. Class I bacteriocins The relative stability of Class II bacteriocins, as well as the absence of post-translational modifications make Class II bacteriocins a good candidate for secretion in yeast. The secretion of a Class II bacteriocin from yeast is the technology pursued in this research.

There have been three previous studies aimed at the secretion of Class II bacteriocins by *S. cerevisiae* (Basanta et al., 2009; Schoeman et al., 1999; Van Reenen et al., 2003). None of the *S. cerevisiae* strains engineered in these studies, summarized in Table 2-1, were able to produce a concentration of bacteriocin that would be considered industrially relevant because the culture supernatants required concentration in order for biological activity to be detected. Concentration of the culture supernatant would not be practical if the yeast is actively secreting the bacteriocin during fermentation.

Table 2-1 Summary of previous attempts to express and secrete bacteriocins in *S. cerevisiae*

Bacteriocin	Vector	Promoter	Signal sequence	Supernatant activity (relative)	Plate activity	Problems suggested	Reference
Pediocin PA1	YEp352	P_{ADH1}	$S_{MF\alpha1}$	Low (concentration required for spot on lawn activity)	Clear zone from 5 day old colony	Low production/secretion	Schoeman, 1999
Plantericin 423	YE_p352	P_{ADH1}	$S_{MF\alpha1}$	Low (concentration required for spot on lawn activity)	Clear zone from 3 day old colony	Cell wall association	Van Reenen, 2003
						Low production/secretion	
Enterocin L50A and L50B	YES_2	P_{GAL1}	$S_{MF\alpha1}$	Moderate (4-11% of producer organism, concentration required for spot on lawn activity)	Not reported	Media interactions (aggregation)	Basanta, 2009
						Proteolysis	
						Interaction with the cell membrane	
						Oxidation of methionine residues	

These previous studies suggested several potential reasons why the bacteriocin activity in the *S. cerevisiae* supernatant was lower than expected. These reasons included low activity of the transcriptional terminator, low secretion efficiency by the secretion signal sequence, low stability or aggregation in the media, proteolysis of the bacteriocin in the culture supernatant, interaction of the bacteriocin with the cell membrane or cell wall, or oxidation of the methionine residues leading to reduced activity. Of these potential problems, only the oxidation of the methionine residues was confirmed by Basanta *et al.* (2009).

The system developed in this study aimed to minimize the problems created by low promoter activity, low secretion efficiency, and the oxidation of methionine residues. The promoter used, P_{GAL1}, is widely regarded as one of the highest-activity promoters in *S. cerevisiae* (K. Lee & DaSilva, 2005). This promotor was also used by Basanta *et al.* for the expression of Enterocin L50A and L50B (2009).The secretion signal sequence used, SS_{MFA1}, is a highly active signal sequence engineered through directed evolution (Rakestraw, Sazinsky, Piatesi, Antipov, & Wittrup, 2009). To avoid a decrease in activity due to the oxidation of the methionine residues, the bacteriocin leucocin A was chosen for this study because it does not contain any oxidizable methionine residues (Hastings et al., 1991). While these potential problems can be managed through the genetic design of the system, the interactions of the bacteriocin with the yeast cell membrane and yeast cell wall, proteolysis of the bacteriocin, and interactions of the bacteriocin with other media components are not as easily controlled, but can be investigated.

2.2 Materials and methods

2.2.1 Culture maintenance

The strains and vectors used in this study are described in Table 2-2 and 2-3. *Escherichia coli* cultures were grown and maintained in Luria-Bertani (LB) broth with appropriate antibiotics (Difco; Becton Dickinson; Sparks, MD). Strains of LAB were grown in All Purpose Tween broth (APT, Difco), with the exception of the large-scale fermentation of *Leuconostoc gelidum* for leucocin A production; the large scale fermentation is described below. *Saccharomyces cerevisiae* cultures were grown in Complete Minimal (CM) Media (Ausubel, 1992) without leucine (CM-Leu) [Yeast Nitrogen Base-AAS (Sigma Aldrich; St. Louis, MO, USA),

ammonium sulfate (Thermo Fisher Scientific; Waltham, MA, USA), dextrose (Thermo Fisher Scientific), amino acids (Sigma Aldrich), and Yeast Extract Peptone (YP) media (BD Yeast Extract (Becton Dickinson), BD Peptone (Thermo Fisher Scientific)] (Ausubel, 1992) with raffinose (YP-Raff) (Sigma Aldrich), dextrose (YP-Glu) (Thermo Fisher Scientific), or galactose (YP-Gal) (Sigma Aldrich) for growth, non-induction, and induction of P_{GAL1} transcription, respectively. All strains were stored at -80°C in 20 % (v/v) glycerol (Sigma Aldrich).

Table 2-2 Organisms used in this study and their relevant characteristics, vectors, and their relevant characteristics

Organism	Name used in this study	Characteristics	Source or reference
Saccharomyces cerevisiae W303-1A		*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ybp1-1*	(B. Thomas & Rothstein, 1989)
Leuconostoc gelidum UAL 187		Leucocin A producer	(Hastings et al., 1991)
Carnobacterium divergens NCIMB 702855		Leucocin A sensitive	(Worobo et al., 1995)
Escherichia coli DH5α™		Molecular cloning	Life Technologies (Grand Island, NY, USA)
Lactobaccillus plantarum G326		Beer spoilage organism, leucocin A sensitive	(Yansanjav et al., 2004)
Saccharomyces cerevisiae W303 YCplac111		Yeast-*E. coli* shuttle vector	Present study
Saccharomyces cerevisiae W303 YCplac111-P_{Gal1}-T_{ADH1}	W303pEmpty	Empty vector	Present study
Saccharomyces cerevisiae W303 YCplac111-P_{Gal1}-GFP-T_{ADH1}	W303pGFP	Empty vector, expression test	Present study
Saccharomyces cerevisiae W303 YCplac111-P_{Gal1}-SS_{MFA1}-LEU-T_{ADH1}	W303pLeucocin	*S. cerevisiae* leucocin A secretion	Present study
Saccharomyces cerevisiae W303 YCplac111-P_{Gal1}-SS_{MFA1}-8AA-LEU-T_{ADH1}	W303p8aaLeucocin	*S. cerevisiae* 8AA-leucocin A fusion secretion	Present study
Saccharomyces cerevisiae W303 YCplac111-P_{Gal1}-SS_{MFA1}-GFP-LEU-T_{ADH1}	W303pGFPLeucocin	*S. cerevisiae* GFP-leucocin A fusion secretion	Present study

Table 2-3 Vectors used in this study, abbreviated names, and their relevant characteristics

Vector	Name used in this study	Characteristics	Source or reference
YCplac111-P_{GAL1}		Yeast-*E. coli* shuttle vector, *ARS, CEN4, LEU2*	(Gietz & Sugino, 1988a)
YCplac111-P_{GAL1}-T_{ADH1}	pEmpty	Empty expression vector	Present study
YCplac111-P_{GAL1}-GFP-T_{ADH1}	pGFP	Empty expression vector, expression test	Present study
YCplac111-P_{GAL1}-SS_{MFA1}-LEU-T_{ADH1}	pLeucocin	*S. cerevisiae* leucocin A secretion	Present study
YCplac111-P_{GAL1}-SS_{MFA1}-8AA-LEU-T_{ADH1}	p8aaLeucocin	*S. cerevisiae* 8AA-leucocin A fusion secretion	Present study
YCplac111-P_{GAL1}-SS_{MFA1}-GFP-LEU-T_{ADH1}	pGFPLeucocin	*S. cerevisiae* GFP-leucocin A fusion secretion	Present study

2.2.2 Genetic manipulation

All of the genetic manipulations described below were performed using *E. coli* DH5α cells (Life Technologies). Restriction digests were performed with enzymes from Life Technologies. Ligations were performed using the Thermo Scientific Rapid Ligation Kit (Thermo Scientific, Pittsburgh, PA, USA). All primers (sequences provided in Table 2-4) were supplied by Integrated DNA Technology (Coralville, IA, USA). Subcloning steps were performed using standard methodology (Sambrook & Russell, 2001). Product sequences were confirmed following each cloning step by Sanger sequencing using the appropriate primers and the BigDye® Terminator Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and sequenced on the ABI 3730 Sequencer (Life Technologies).

The vector YCplac111-P_{GAL1} was obtained from the Stuart Lab (University of Alberta, Edmonton, AB, Canada). The sequence for the transcriptional terminator T_{ADH1} was then amplified from total DNA extract of *S. cerevisiae* and cloned into the *Bam*H1 and *Eco*R1 sites of YCplac111-P_{GAL1} to create the vector YCplac111-P_{Gal1}-T_{ADH1} (pEmpty). The genes for GFP, SS_{MFA1}-LEU, SS_{MFA1}-8AA-LEU, SS_{MFA1}-GFP-LEU, were then amplified from DNA templates synthesized by BioBasic Inc. (Markham, ON, Canada) and GenScript (Piscataway, NJ, USA) and cloned into the *Bam*H1 and *Kpn*1 sites of the pEmpty vector to create the plasmids pGFP, pLeucocin, p8aaLeucocin, and pGFPLeucocin, respectively.

Following sequence confirmation, the plasmids pEmpty, pGFP, pLeucocin, p8aaLeucocin, and pGFPLeucocin were transformed into *S. cerevisiae* W303 cells using a DMSO assisted yeast transformation protocol (Hill, Donald, & Griffiths, 1991) and plated on CM-Leu media. The strains will be referred to as W303pEmpty, W303pGFP, W303pLeucocin, W303p8aaLeucocin, and W303pGFPLeucocin, respectively.

Table 2-4 PCR Primers used in this study. Restriction sites are italicized

Gene product	Template		Primer sequence (5' to 3')
T_{ADH1}	*S. cerevisiae* total DNA	N	CTA*GGTACC*TAATAAGCGAATTTCTTATGTTTATG
		C	CAT*GAATTC*GGGAGCGATTTGC
GFP	MFA1-GFP-Leu (Figure X-1)	N	CATAC*GGATCC*ATGTCTAAGGGTTGAAGA
		C	CTTAC*GGTACC*TTAACGTTTATCCAGTTGG
SS_{MFA1}-LEU	MFA1-Leu-GFP (Figure X-2)	N	CATAC*GGATCC*ATGAGGTTCCCTTC
		C	CTTAC*GGTACC*TTACCAAAAACCGTTTCC
SS_{MFA1}-8AA-LEU	MFA1-8AA-Leu (Figure X-3)	N	CATAC*GGATCC*ATGTCTAAGGGTTGAAGA
		C	CAA*GGTACC*TCACCAGAAACCGTT
SS_{MFA1}-GFP-LEU	MFA1-GFP-Leu (Figure X-1)	N	CATAC*GGATCC*ATGAGGTTCCCTTC
		C	CTTAC*GGTACC*TTACCAAAAGCCATTTCC

2.2.3 Expression of proteins in yeast

Transformed colonies were selected from CM-Leu plates and grown for 40 h in 5 mL YP-Raff, at 200 rpm and 30°C. The overnight culture was used to inoculate a 250 mL shake flask containing 100 mL of YP-Raff, which was grown 24 h at 200 rpm and 30°C. The OD_{600} of the cultures was measured. A volume of the cultures was used to normalize all cultures to OD_{600} = 1.0. The cultures were aseptically centrifuged (1 500 x *g*, 5 min), pellets were washed with 50 mL MilliQ water, centrifuged (1 500 x *g*, 5 min), and resuspended in 100 mL of YP-Gal media. Cultures were shaken for 24 h at 200 rpm and 30°C.

For spot-on-lawn assays, cultures were centrifuged (1 500 x *g*, 5 min). The supernatant was removed and heat-treated for 20 min at 60°C to kill the yeast. The supernatant was freeze-dried to concentrate and resuspended in Milli-Q water prior to use.

For SDS-PAGE and Western blot, cell pellets from 0.5 mL culture taken 24 h after induction were suspended in 100 µL of SDS load dye [100 mM Tris pH 6.8 (Sigma-Adrich), 4% SDS (Sigma-Aldrich), 20% Glycerol (Fisher), 0.2% Bromophenol Blue (Sigma-Aldrich), 10% 2-mercaptoethanol (Sigma-Aldrich)]. Twenty µL of the resuspended pellets were separated on two 15% SDS-polyacrylamide gels, one of which was subsequently stained with Coomassie Brilliant Blue for visualization of proteins. The proteins from the second gel were transferred to an Immobilon-P PVDF membrane (EMD Millipore, Darmstadt, Germany) and blotted with an anti-GFP antibody (GE Life Sciences, Piscataway, NJ).

2.2.4 Spot on lawn assays

A petri dish containing APT (Difco) agar (Fisher) was overlaid with 5 mL of APT soft agar [0.75 % agar (w/v)] inoculated with 50 µL of an overnight culture of the strain being tested. The agar was allowed to solidify, and then 10 µL of the 1 600 AU/mL leucocin A solution was spotted on the plate. The plate was allowed to dry under a biosafety cabinet for approximately 15 min, and the plate was incubated overnight at 25°C.

2.2.5 Purification of leucocin A from L. gelidum

Leucocin A was purified using a modified version of the method described by Hastings & Stiles (1991). A Minifors 5 L fermenter (Infors HT; Bottmingen CH) containing 3.5 L of CAA media (Hastings et al., 1991) was inoculated with 250 mL of an overnight culture of *Leuconostoc gelidum* UAL187 and incubated under N_2 at 25°C, with 100 rpm agitation and a maintained pH of 6.0. Twenty five h after inoculation, the culture supernatant was harvested by centrifugation at 6 000 x *g* for 15 min. The supernatant was transferred to flasks and incubated at 60 °C for 30 min to kill the bacteria. Following incubation, the supernatant was cooled to room temperature and ammonium sulfate (Fisher) precipitation to 70% (w/v) saturation was performed. The ammonium sulfate product was stirred overnight, and the precipitate was recovered by centrifugation at 6 000 x *g* for 15 min. The pellet was resuspended in 50 mL 6 M urea (Fisher) and 10 mM glycine (Sigma-Aldrich), pH 2.5, and then applied to Amberlite XAD-2 (Sigma-Aldrich) resin, washed with 25% and 45% ethanol in 0.1% trifluoroacetic acid (Sigma-Aldrich), and eluted with 75% ethanol in 0.1% trifluoroacetic acid. Fractions were tested for antimicrobial activity against *Carnobacterium divergens* NCIMB 702855 using a spot-on-lawn assay and then pooled and

evaporated to dryness by rotary evaporation (Büchi; Flawil, CH). The residue was resuspended in 2.5 mL MilliQ water and loaded onto a Superdex Peptide GL column (GE Healthcare; Uppsala, SE) connected to an Äktapurifier FPLC system (GE Healthcare) with 137 mM sodium chloride (Fisher) and 12 mM phosphate, pH 7.4 (Sigma-Aldrich) as the elution buffer, at a flow rate of 1 mL/min. 1 mL fractions were collected and pooled based on antimicrobial activity. The pooled solution was stored at 4 °C.

2.2.6 Large scale concentration and purification of leucocin A from yeast supernatant

Leucocin A was expressed as per section 2.2.3, except volumes were scaled up. 4 x 5 mL of YP-Raff media were inoculated with a single colony and incubated at 30°C and 200 rpm for 40 h. 4 x 50 mL of YP-Raff media were inoculated with the 5 mL culture and incubated at 30°C and 200 rpm for 24 h. 4 x 250 mL YP-Raff in 500 mL shake flasks were inoculated with the 50 mL cultures and incubated at 30 C and 200 rpm for 24 h. The OD_{600} of the cultures was measured. A volume of the cultures was removed to normalize the OD_{600} to 1.0, and the cultures were aseptically centrifuged (1 500 x *g*, 5 min), pellets were washed with 200 mL MilliQ water, centrifuged (1 500 x *g*, 5 min) and resuspended in 250 mL YP-Gal media. Cultures were shaken for 24 h at 200 rpm and 30°C. The cultures were centrifuged (1 500 x *g*, 5 min) and the supernatant was removed and heat treated at 60°C for 20 min. The supernatant was then split into two 500 mL fractions for butanol extraction and ammonium sulfate precipitation.

One 500 mL fraction was extracted 3 times with 500 mL butanol (Fisher). The butanol phase was concentrated by rotoevaporation (to dryness) and the dry pellet was resuspended in 10 mL MilliQ. Bacteriocin activity of the solution was assayed using a spot on lawn assay as described in section 2.2.4.

Ammonium sulfate (239 g, Fisher) was slowly added to the second 500 mL fraction of supernatant. The suspension was stirred overnight, and then centrifuged at 6 000 x *g* for 15 min. The pellet was resuspended in 50 mL 6 M urea (Fisher) and 10 mM glycine (Sigma-Aldrich), pH 2.5 and then applied to Amberlite XAD-2 (Sigma-Aldrich) resin, washed with 25% and 45% ethanol in 0.1% trifluoroacetic acid (Sigma-Aldrich) and eluted with 75% ethanol in 0.1% trifluoroacetic acid. Fractions were tested for antimicrobial activity against *C. divergens* NCIMB 702855.

2.2.7 Incubation of purified leucocin A with yeast spheroplasts

Five hundred mL of YP-Glu media was inoculated with 50 mL of an *S. cerevisiae* W303 culture and incubated at 200 rpm and 30°C for 4 h. The culture was split in two and centrifuged (1 500 x *g*, 5 min). Both halves were subjected to the following spheroplasting protocol, but only one half was treated with lyticase. The pellets were washed with four volumes of water, centrifuged (1 500 x *g*, 5 min), the supernatant was then decanted and one volume of spheroplast buffer (67 mM phosphate (Fisher), pH 7.5) with 30 mM DTT (Sigma) was added. The suspension was incubated 15 min at 25°C with gentle shaking. The suspension was centrifuged (1 500 x *g*, 5 min), the supernatant was decanted, and 3 volumes of lyticase buffer with 1 mM DTT was added. 600 U of lyticase (Lyticase from *Arthrobacter luteus*, Sigma) was added to the spheroplast tube. The suspension was incubated 1 h at 30°C. The formation of spheroplasts was monitored by examination of the shape of the cells after washing and for lysis after exposure to a 5% SDS (Sigma) solution. The suspensions were centrifuged (1 500 x *g*, 5 min) and the supernatant was decanted. The cells were washed 3 times with 3 volumes of lyticase buffer.

Spheroplasts or whole cells (0, 50, or 100 µL) were then added to a solution containing 20 AU of purified leucocin A and the volume was brought up to 200 µL with lyticase buffer. The suspension was incubated at 25°C for 20 minutes. The mixture was centrifuged (1 500 x *g*, 5 min) and the supernatant was removed and used for spot on lawn assays as per section 2.2.4, using *Carnobacterium divergens* NCIMB 702855 as the test strain. The experiment was performed in triplicate.

2.2.8 Addition of lyticase and alcohol to leucocin-A secreting yeast culture

Triplicate cultures of W303pLeucocin were prepared as per section 2.2.3 and split into 5 mL aliquots. Each aliquot was treated with or without 500 U of lyticase (from *Arthrobacter luteus,* Sigma) in combination with or without 500 µL ethanol (Fisher), 1000 µL ethanol, or 1000 µL isopropanol (Fisher) to give final percentages of 9%, 17%, and 17%, respectively. The aliquots were incubated at 30°C and 200 rpm for one hour. The aliquots were centrifuged (1 500 x *g*, 5 min) and the supernatant was decanted. The supernatant was heated for 20 min at 60°C, then the supernatant was freeze-dried. The freeze-dried pellets were resuspended in 100 µL of

MilliQ water and 5 µL was spotted on a lawn of *C. divergens* NCIMB 702855 as described in section 2.2.4.

2.2.9 Proteinase co-spot experiment

A supernatant of W303pLeucocin was prepared as described in section 2.2.3. The supernatant was concentrated 50X by freeze-drying. The supernatant was spotted alone on a plate or co-spotted 1 cm from 5 µL of 1 mg/mL trypsin (from bovine pancreas, Sigma) as described in the spot on lawn procedure in section 2.2.4.

2.2.10 Co-culture of La. plantarum with yeast

The W303pEmpty, W303pLeucocin, W303p8aaLeucocin were grown from single colonies in 5 mL YP-Raff media for 48 h at 30°C and 200 rpm. These cultures were used to inoculate a 250 mL culture of the same media, which was incubated for 24h at 30°C and 200 rpm. These triplicate cultures were normalize to OD_{600}=1.0 and used to inoculate 250 mL flasks of YP-Gal media. These flasks were incubated at 30°C and 200 rpm for one hour. The flasks containing the yeast cultures, as well as flasks containing the YP-Gal media, and the YP-Gal media plus 1% ethanol (Fisher) were inoculated to a final cell density of 2 X 10^4 CFU/mL *La. plantarum* G326 overnight culture. The flasks were sampled 0, 6, 18, 24, and 48 hours after *La. plantarum* inoculation and a full dilution series was plated on APT + Fungizone (Life Technologies) plates.

2.3 Results

2.3.1 Plasmid assembly and test of expression system

YCplac111 plasmids containing the yeast *GAL1* promoter (P_{GAL1}) (West, Chen, Puntz, Butler, & Banerjee, 1987) were obtained and the *ADH1* transcriptional terminator (T_{ADH1}) (Denis, Ferguson, & Young, 1983) was cloned downstream to create pEmpty. Confirmation of the desired plasmid was achieved through restriction digest analysis (Figure 2-1) and subsequent sequencing. Between the promoter and transcriptional terminator, the genes for three protein fusions between the mating factor α 1 secretion signal sequence (SS_{MFA1}) (Rakestraw et al.,

2009), leucocin A (LEU) (Hastings et al., 1991), and green fluorescent protein (GFP) (Prasher, Eckenrode, Ward, Prendergast, & Cormier, 1992) were cloned into pEmpty: SS_{MFA1}-LEU, GFP, SS_{MFA1}-GFP-LEU to create the plasmids pLeucocin, pGFP, and pGFPLeucocin. The restriction digest analysis for these three gene products cloned into pEmpty are shown in Figure 2-2 and were confirmed by sequence analysis. The pEmpty constructs were used for expression experiments because P_{GAL1} allows for inducible, high-level expression (K. Lee & DaSilva, 2005), ideal for testing the maximum output of the expression and secretion system. pEmpty, pGFP, pLeucocin, and pGFPLeucocin were then transformed into *S. cerevisiae* W303-1A cells to create the strains W303pEmpty, W303pGFP, W303pLeucocin, and W303pGFPLeucocin.

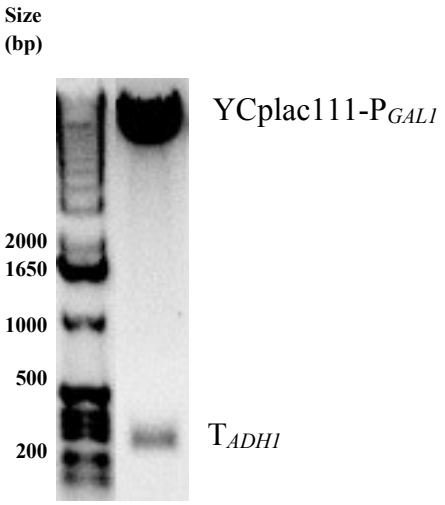


Figure 2-1 Results of transcriptional terminator cloning into YCplac111-P_{GAL1} to create pEmpty

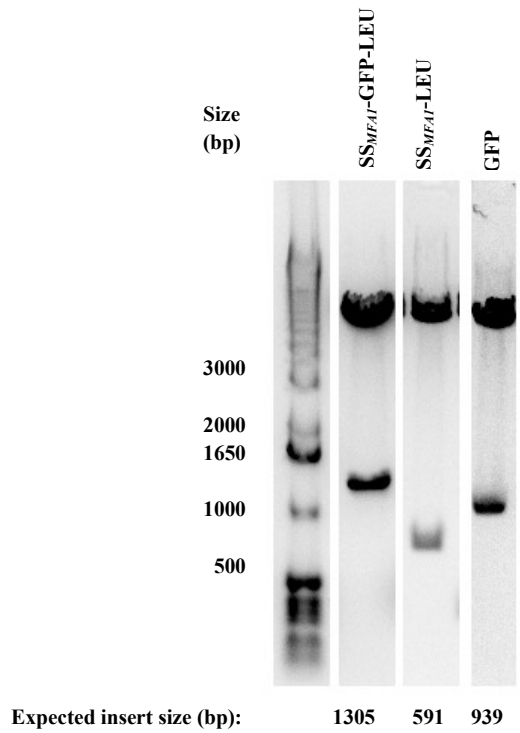


Figure 2-2 Results of test digest of leucocin fusion proteins cloned into pEmpty expression system to create pGFPLeucocin, pLeucocin, and pGFP. Expected fragments sizes are for the construct with the C-terminal ADH1transcriptional terminator sequence.

The expression of GFP in the construct YCplac111-P$_{GAL1}$-GFP-T$_{ADH1}$ was verified using Western blotting with an anti-GFP antibody (Figure 2-3). Under the expression conditions used, the whole cell did not produce a prominent band in the SDS-PAGE system, but did produce a single, clear band in the Western blot. This indicated that the protein is being expressed intracellularly under the control of the transcriptional promoter and terminator. This indicates that protein expression is relatively low compared with organisms such as *E. coli*, which generally produces a prominent protein band in SDS-PAGE when expression is induced with a highly active promoter.

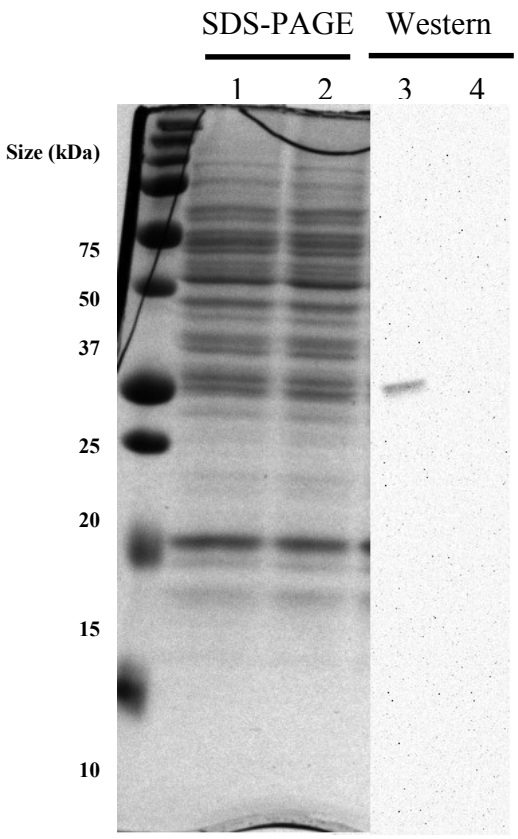


Figure 2-3 SDS-PAGE (Lanes 1, 2) and Western Blot (Lanes 3, 4) of TCA-extracted protein from W303pGFP cells (Lanes 1, 3) and W303pEmpty cells (Lanes 2, 4).

2.3.2 Expression and detection of leucocin A

Because of the difficulties with immunochemical detection of Class IIa bacteriocins including leucocin A (D. Diep & Nes, 2002), detection of the protein could only be performed using indirect Western blotting against the GFP-leucocin A fusions with an anti-GFP antibody, or using spot on lawn assays to detect bacteriocin activity. The strain W303pLeucocin was used to test the expression and secretion of leucocin A. When spot on lawn assays were performed, the activity of the W303pLeucocin supernatant was compared with the activity of the W303pEmpty strain, in order to control for any clearing effect created by other components of the yeast cell supernatant.

In cultures of W303pLeucocin, bacteriocin activity was not detected in the supernatant of cultures at time points 0 – 48 hours after galactose addition (data not shown). However, when the supernatant was concentrated 50X by freeze-drying, 10 µL of the concentrated supernatant gave a faint zone of inhibition in a spot-on-lawn assay (Figure 2-4). This is a much lower production level than the producer organism, *Leuconostoc gelidum* UAL187; a 10 µL spot of an overnight *L. gelidum* UAL187 culture supernatant typically gives a clearly defined 1.0 cm zone of inhibition against this strain (data not shown).

Although the culture supernatant of a strain containing the pEmpty also gave a very faint zone of clearing on the plate, the zone of clearing created by the W303pLeucocin strain was consistently larger with sharper edges. The spot from the W303pLeucocin strain also exhibited sensitivity to protease treatment. When the concentrated supernatant from the W303pLeucocin strain was co-spotted side by side with trypsin, the spot exhibited a half-moon shaped zone of clearing consistent with the proteolytic inactivation of the bacteriocin (Figure 2-5). These findings suggest that the bacteriocin was being expressed and secreted from the cells and is responsible for the anti-microbial activity of the supernatant. While further concentration and purification of the bacteriocin was attempted in order to obtain mass spectrometry data confirming the presence of leucocin A, we were unable to obtain a purified sample with a high enough concentration and activity to successfully perform mass spectrometry.

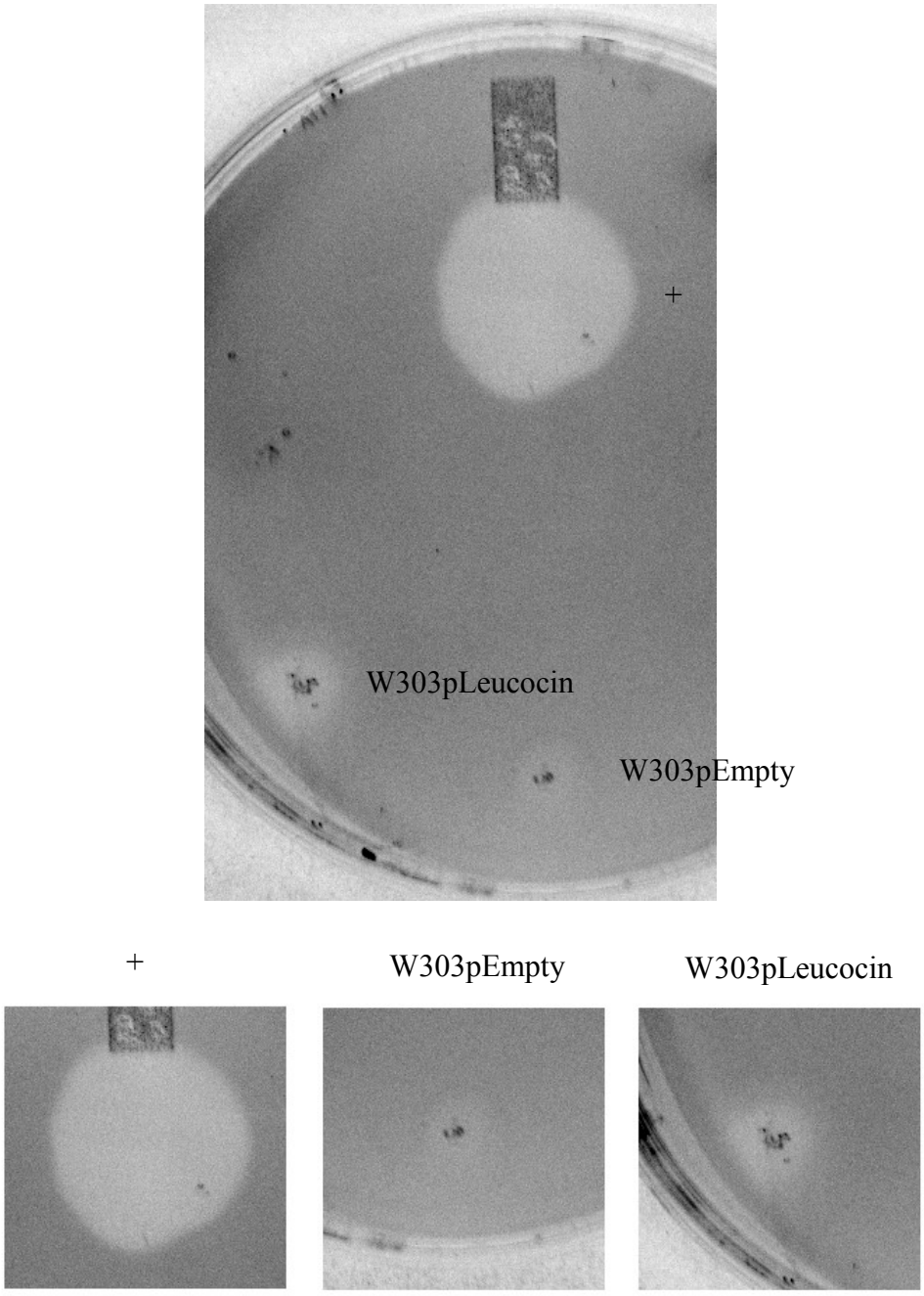


Figure 2-4 Spot on lawn assay (lawn *Carnobacterium divergens* NCIMB 702855) using purified leucocin A (+), 50X concentrated culture supernatant of W3030pEmpty, or 50X concentrated culture supernatant of W303pLeucocin).

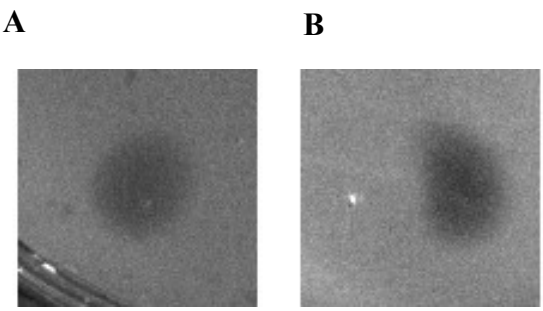


Figure 2-5 Spot on lawn assay of 50X concentrated W303pLeucocin supernatant without (A) and with (B) co-spotting with trypsin.

2.3.3 Improvement of leucocin A excretion

One hypothesis for the low levels of bacteriocin activity in the culture supernatant is that the protein is being secreted out of the cell, but not diffusing past the robust yeast cell wall. Venturini *et al.* (1997) found that the 8 amino acid sequence FPTALVRR on the N-terminus of *S. cerevisiae* Glucoamylase II (encoded by the gene *STA2*) improved the excretion of the enzyme past the cell wall and into the culture medium. This 8 amino acid sequence (8AA) was added to the construct YCplac111-P_{GAL1}-SS_{MFA1}-LEU-T_{ADH1} to make YCplac111-P_{GAL1}-S_{MFA1}-8AA-LEU-T_{ADH1} (Figure 2-6) (p8aaLeucocin).

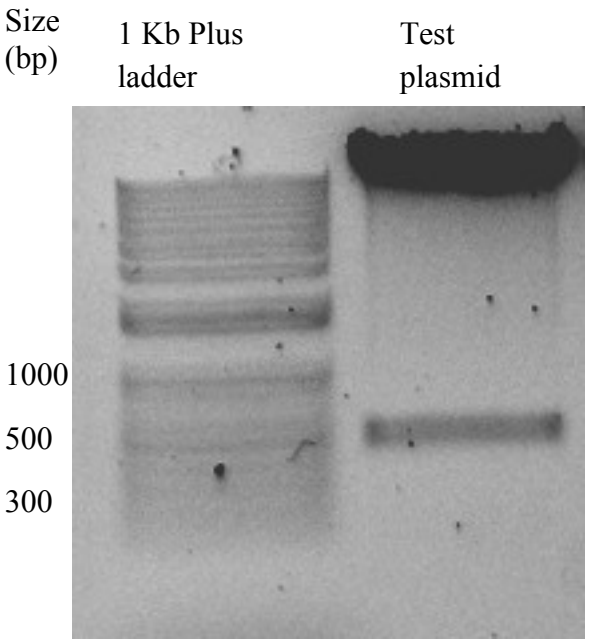


Figure 2-6 Results of test digest (*Bam*H1 and *Kpn*1) of SS_{MFA1}-8AA-LEU cloned into YCplac111-P_{GAL1}-T_{ADH1}, expected fragment size: 7539 bp (vector), 393 bp (insert).

The culture supernatant of the W303p8aaLeucocin strain was concentrated by freeze-drying and spotted on a lawn of *C. divergens* NCIMB 702855. The strain with the 8AA addition consistently gave a larger zone of clearing than the strain without the 8AA addition (Figure 2-7). This indicates that the 8AA addition may be aiding the export of leucocin A beyond the cell wall.

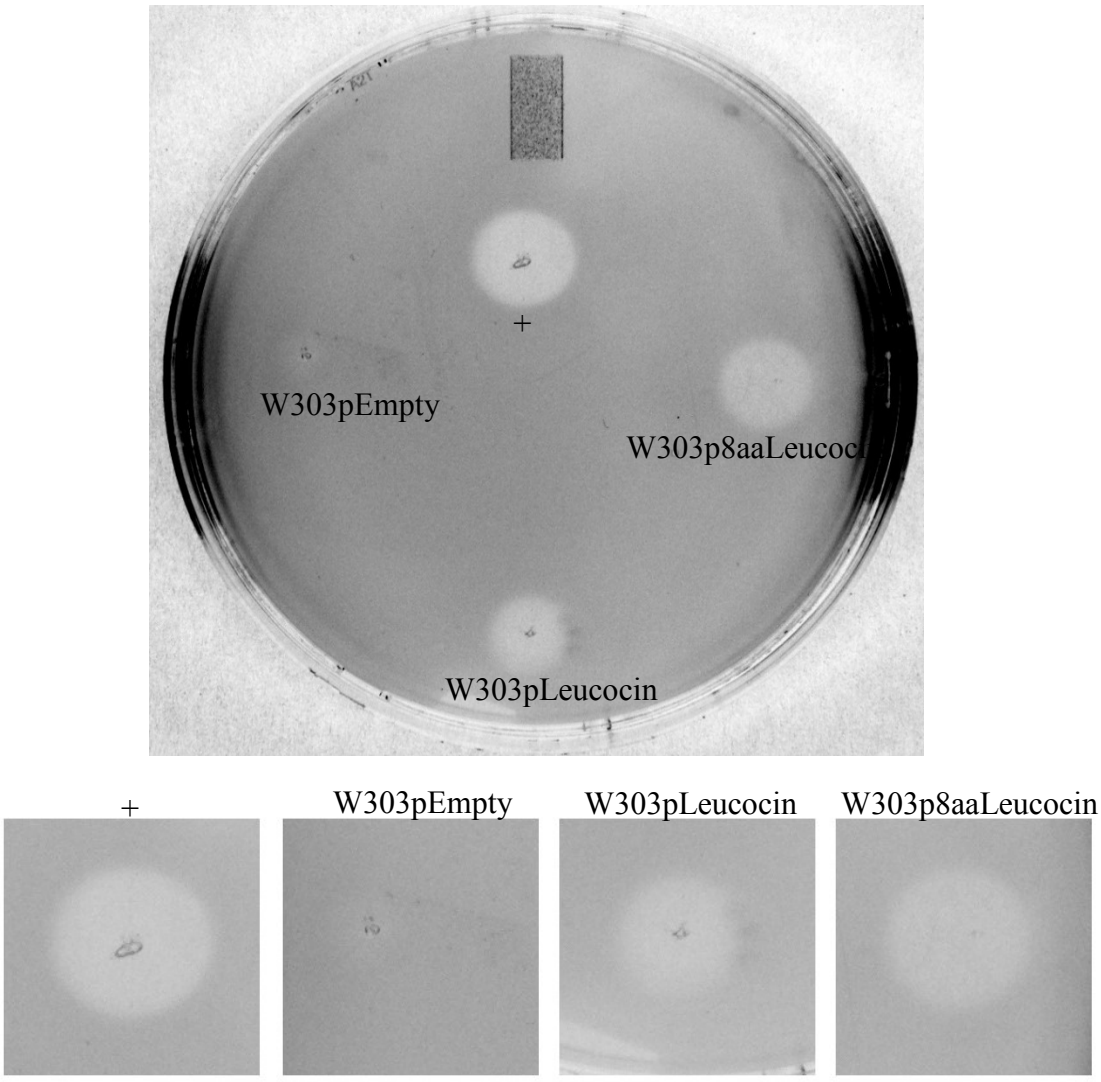


Figure 2-7 Spot on lawn assay of purified leucocin A (1 AU) (+), 50X concentrated supernatant from W303pEmpty, W303pLeucocin, W303p8aaLeucocin.

2.3.4 Interactions between the yeast cell membrane and leucocin A

Another potential reason for low apparent bacteriocin activity in the cell supernatant is an interaction between the bacteriocin and the yeast cellular membrane. To probe this interaction, purified leucocin A was incubated with either intact yeast cells or spheroplasts. The intact yeast cells were carried through the spheroplasting methodology, but were not treated with lyticase. The spheroplasts titrated the bacteriocin activity from the supernatant; as more spheroplasts were

added, the bacteriocin activity of the supernatant went down (Figure 2-8). The bacteriocin activity of the leucocin A solution incubated with the whole yeast cells remained statistically constant. This suggests that the bacteriocin interacts directly with the cell membrane of the yeast cell.

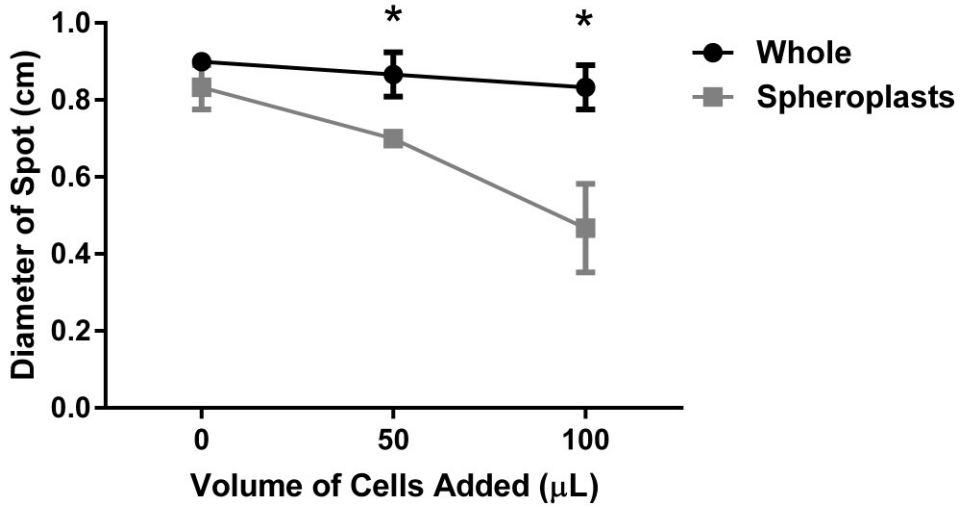


Figure 2-8 Bacteriocin activity of purified leucocin A solution following incubation with whole yeast cells (whole) or yeast spheroplasts (spheroplasts). Error bars show standard deviation, n = 3. Asterisk indicates statistically significant difference (t-test), $p \leq 0.05$.

To further study the interaction between leucocin A, the yeast cell membrane, and the yeast cell wall, W303pLeucocin was treated with alcohol to disrupt the cell membrane and lyticase to disrupt the cell wall after 24 h of induced expression. Short chain alcohols are known to increase membrane fluidity (Alexandre E, Berlot, & Charpentier, 1994; Ly & Longo, 2004). After treatment, the supernatants dried and resuspended at a 50X concentration and spotted on a lawn. Alcohol treatments increased the size of the zone of clearing on the plates (Figure 2-9). This increase was more pronounced when lyticase was used in tandem. These data indicate that both the cell wall and the cell membrane may play a role in preventing leucocin secretion from the cell membrane. As the concentration and hydrophobicity of the alcohol increased, the bacteriocin activity of the supernatant increased. This suggests that the alcohol is disrupting a hydrophobic interaction between the yeast cell membrane and the bacteriocin.

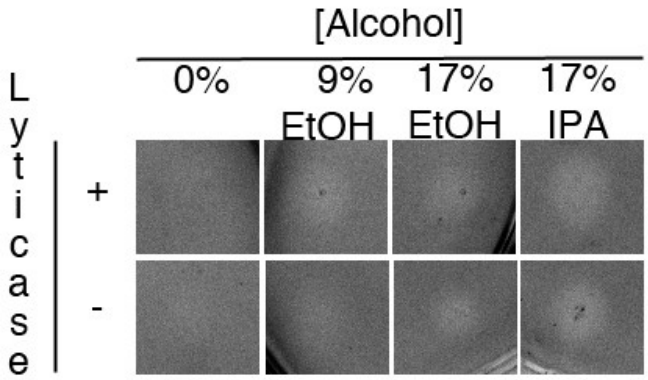


Figure 2-9 Bacteriocin activity of yeast supernatant after treatment of cells with lyticase and/or alcohol. EtOH, Ethanol, IPA, Isopropyl alcohol.

2.3.5 In silico analysis of post-translational modification of leucocin A

The potential for glycosylation during the protein export process was examined. *S. cerevisiae* has the machinery for both N-glycosylation and O-glycosylation of peptides; the N-glycosylation site is an asparagine residue proceeded by the sequence X-Ser/Thr, where X is any amino acid (Lehle & Bause, 1984). O-glycosylation can occur on any Ser or Thr residue and is likely dependent on protein secondary and tertiary structure (Lehle & Bause, 1984). The putative glycosylation sites for the SS_{MFA1}-LEU protein are presented in Figure 2-10. Potential N and O-glycosylation sites occur on the protein.

*MRFPSIFTAVLFAASSALAAPANTTTEDETAQIPAEAVIDYSDLEGDFDAAALPLSNSTNNG LSSTNTTIASIAAKEEGVQLDKR***KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGG NGFW**

Figure 2-10 Primary sequence of SS_{MFA1} (purple, italicized) LEU (blue, bold). Potential N-gycosylation sites (Asn-X-Ser/Thr) are highlighted in yellow. Potential O-glycosylation sites (Ser/Thr) are underlined.

Attempts were made to examine if leucocin and GFP-leucocin fusion proteins expressed in W303pLeococin and W303pGFPLeucocin using an endoglycosidase-Western blot analysis. Results of the endoglycosidase-Western blot analysis were inconclusive (data not shown). It is not possible to rule out glycosylation of the protein as a factor decreasing the activity of the protein in the supernatant.

2.3.6 Attempts to purify leucocin A from culture media

In order to clearly elucidate whether leucocin A is being excreted as a full length, unmodified protein, attempts were made to extract, concentrate and purify the protein. Butanol extraction and ammonium sulfate precipitation were both attempted on a large volume of W303pLeucocin culture supernatant. Neither preparation produced a significant amount of activity that suggested that a protein mass by mass spectrometry could be obtained.

2.3.7 Application of the system against a beer spoilage organism in liquid culture

Although the bacteriocin activity in the yeast cell supernatant is much lower than a typical *L. gelidum* culture and our findings indicate that this could be due to an interaction between the bacteriocin and both the cell wall and cell membrane of the yeast cells, it may be possible that the bacteriocin could preferentially interact with lactic acid bacteria in a mixed culture situation. To probe this, we co-cultured yeast strains W3030pEmpty, W303pLeucocin, and W303p8aaLeucocin with *Lactobacillus plantarum* G326, a beer spoilage strain, and measured the growth of the *La. plantarum* strain on selective media (Figure 2-11). A 2% ethanol control was also included to control for the maximal amount of ethanol that could be produced by the yeast strains from the media, as well as to potentially increase the release of bacteriocin in from the yeast cells, as was demonstrated in Figure 2-9. The *La. plantarum* strain was not inhibited by 2% alcohol, compared with a control containing only *La. plantarum* and YP-Gal media. The yeast cells secreting leucocin A or 8AA-leucocin A were not able to control *La. plantarum* growth better than the control strain of yeast that contained the empty vector.

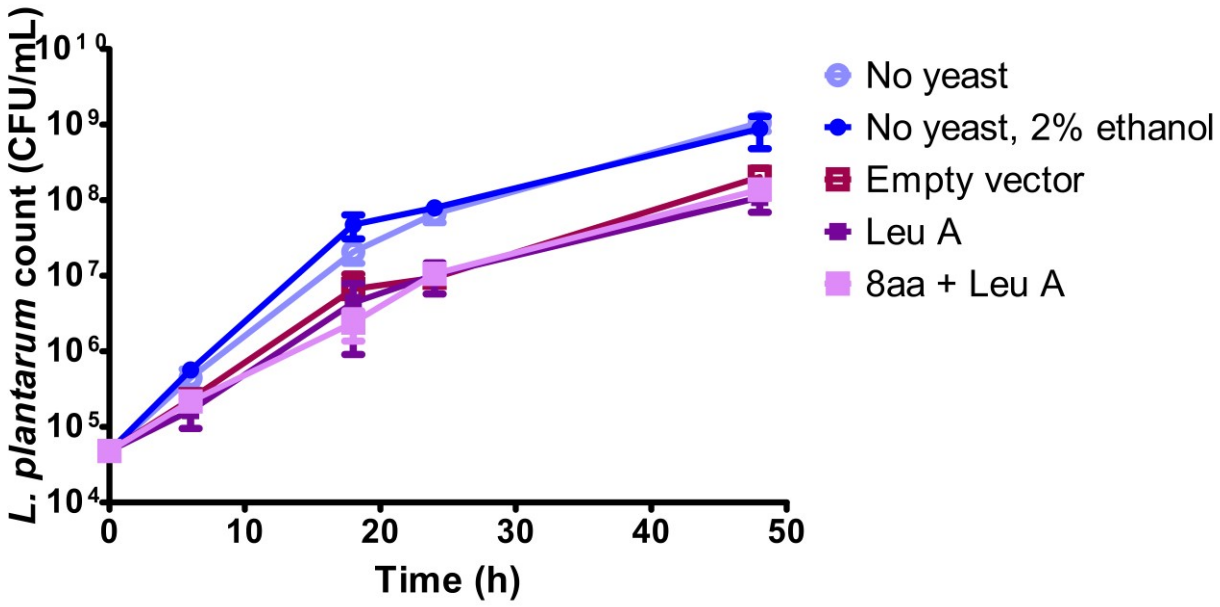


Figure 2-11 Cell counts of *La. plantarum* in YP-GAL liquid media with/without additives and engineered yeast. Yeast was grown for 24 h, protein expression was induced for 24 h, and then the yeast culture was added to the *La. plantarum* culture at t=0, where appropriate. Error bars: standard deviation, n = 3.

2.4 Discussion

In this study, we were able to successfully engineer yeast cells that secreted the bacteriocin leucocin A. The secretion was improved with the addition of an N-terminal 8 amino acid peptide sequence. Despite the improvement, this yeast strain still secreted the bacteriocin at low levels relative to the bacterial producer strain *Leuconostoc gelidum* UAL187, and was unable to prevent the growth of an *La. plantarum* beer spoilage strain in a co-culture experiment.

The relatively low levels of bacteriocin activity that we achieved are not unprecedented. Groups that have previously tried to express and secrete bacteriocins in *Saccharomyces cerevisiae* observed similar low levels of bacteriocin activity in the culture supernatant (Basanta et al., 2009; Schoeman et al., 1999; Van Reenen et al., 2003). These groups attributed the low activity levels to a number of possible problems (summarized in Table 2-1), including sub-optimal expression and secretion due to promoter and signal sequence choice (Schoeman et al.,

1999; Van Reenen et al., 2003), potential protein glycosylation and cell wall association (Schoeman et al., 1999), proteolytic degradation or aggregation of the bacteriocin monomers in the liquid media, inactivation of the bacteriocin due to oxidation of methionine groups, and interaction of the bacteriocin with the yeast cell membrane (Basanta et al., 2009). Of these proposed problems, only the oxidation of the methionine groups was confirmed as an issue affecting the activity of enterocin L50A and L50B by Basanta et al (2009). Because leucocin A does not contain methionine residues, there is no possibility that oxidation of methionine contributed to the low activity.

With the goal of maximizing protein expression and secretion in this project, we chose a highly active promoter and secretion signal sequence as well as codon-optimized the DNA sequence for *S. cerevisiae*. The *GAL1* promoter was chosen for this project because is generally known as the most highly active inducible promoter used in *S. cerevisiae* (K. Lee & DaSilva, 2005). The secretory leader peptide used in this study was engineered by Rakestraw *et al.* (2009) to have significantly higher secretory activity than the wild-type *MFα1* leader sequence. This genetically optimized combination of promoter and leader peptide should have maximized the bacteriocin output from the cell; low activity in the supernatant is likely caused by different factors that are discussed below.

Based on the result that purified leucocin A interacts with spheroplasts and not intact yeast cells, it is likely that, following secretion, the bacteriocin is interacting with the cell membrane of the yeast cells. Bacteriocins have previously been shown to interact with model membrane systems of *S. cerevisiae*, first through an ionic interaction with phospholipids, followed by a hydrophobic interaction (Lopes et al., 2009). More recent research has shown that this interaction is also dependent on electrostatic interactions between the bacteriocin and glycosylated membrane proteins (Sand, Nissen-Meyer, Sand, & Haug, 2013). Our findings that alcohols can release bacteriocins from the cell membrane and that stronger or more concentrated alcohols release more bacteriocin suggests the involvement of a hydrophobic interaction involved and the hydrophobic interaction, which is interrupted by the presence of alcohol.

Despite the finding that bacteriocins interact with the yeast cell membrane, bacteriocins have been used effectively to control lactic acid bacteria contamination in laboratory studies of fuel ethanol production without lowering yeast cell counts or ethanol yield at levels ranging from

3 – 5 mg/L (M. A. Franchi, Tribst, & Cristianini, 2012; Peng et al., 2012). Based on our result that intact yeast cells do not interact with leucocin A and that adding lyticase, a cell wall hydrolyzing enzyme, to leucocin-producing cells increases the bacteriocin activity of the culture supernatant, it is likely that the cell wall prevents bacteriocin diffusion both into and out of the yeast cell. Furthermore, it is likely that the interaction of bacteriocins with the cell membrane also plays a large role in preventing efficient bacteriocin secretion.

Another possible explanation for low bacteriocin activity in this system is that the bacteriocin is glycosylated prior to cell export and that the glycosylation affects the activity of the protein (Schoeman et al., 1999). Our results from the deglycosylation experiment were inconclusive and further study should be done because the bacteriocin contains potential glycosylation sites. Glycosylation could be confirmed by MALDI experiments if an appropriate amount of bacteriocin can be purified.

Proteolysis of the bacteriocin in the cell supernatant is another possible reason for the low activity. Basanta *et al.* (2009) suggested that dying yeast cells could release vacuolar proteases that digest the bacteriocins in the culture supernatant. Yeast cells have also been shown to excrete proteases in response to extracellular protein in media (Kurucova, Farkasova, Varecka, & Simkovic, 2009). These findings are especially significant when the conditions of very high gravity fermentation for ethanol production are considered; high concentrations of grain (up to 30%), and therefore protein, in the fermentation media could heavily stimulate the excretion of proteases. Because of the potential presence of proteases in the culture media, proteolysis may play a role in decreasing the bacteriocin activity in the supernatant of bacteriocin-secreting yeast. A future direction for this work could be to express the protein in protease-deficient yeast.

Due to their hydrophobic nature, it is also possible that secreted bacteriocins may be aggregating in the culture media. This is a difficult hypothesis to test. However, if this were a large problem, bacteriocins would aggregate during storage, especially in solutions that contain no detergent. In this study, purified leucocin A was stored in phosphate buffered saline at 4 °C for long periods of time without a large depreciation in activity. Although aggregation in the supernatant may play some role in the low activity, it is likely that the major losses can be attributed to cell membrane or cell wall interactions.

Chapter 3: Towards expression and cyclization of carnocyclin A, a cyclic bacteriocin, in *Escherichia coli*

3.1 Introduction

Cyclic peptides and proteins are molecules where the peptide backbone is cyclized by a covalent peptide bond between the N and C terminus. This cyclization protects proteins and peptides from proteolysis by exoproteases (R. J. Clark, Akcan, Kaas, Daly, & Craik, 2012; R. Clark et al., 2005). It has also been suggested that cyclization stabilizes the three dimensional protein structure by constraining the movement of the N and C termini of the protein, which would otherwise move more freely (Aboye & Camarero, 2012). Constraining the topology of the protein can increase the precision of receptor-binding if the protein is biologically active (R. J. Clark et al., 2012; R. Clark et al., 2005). Thus, the cyclization of peptides and proteins has become an increasingly important area of study.

Cyclic peptides occur naturally in bacteria, fungi, plants, and animals (Aboye & Camarero, 2012). Class IV bacteriocins are ribosomally synthesized, head-to-tail cyclized bacteriocins that are produced in Gram-positive bacteria. Members of this class of bacteriocins have been called "close to perfection" for their broad spectrum of activity and relative stability (Sanchez-Hidalgo et al., 2011), and are therefore attractive industrial molecules, specifically for food preservation applications. Thirteen Class IV bacteriocins have been discovered and have been divided into two groups based on primary structure and chemical characteristics. Group i bacteriocins are cationic and have a high pI (>9.0), while those of Group ii are highly hydrophobic and have a lower pI (<7.0). Carnocyclin A, the bacteriocin used in this study, is a member of Group i.

Carnocyclin A was chosen for this study mainly due to the local expertise on the properties of the bacteriocin, as the bacteriocin was discovered and characterized by University of Alberta researchers (Martin-Visscher et al., 2008). This bacteriocin has activity against several strains of *Lactobacillus* and *Lactococcus* (Martin-Visscher et al., 2008), so it could potentially be used against spoilage organisms in the bioethanol industry. Additionally, carnocyclin A also has

anti-Listerial properties, which make it desirable for food applications (Martin-Visscher et al., 2008).

Ten genes have been identified that may play a role in the production of and immunity to carnocyclin A in the producer organism *Carnobacterium maltaromaticum* UAL307 (Belkum, Martin-Visscher, & Vederas, 2010). Six of these genes have been deemed essential for production: CclA, the structural gene for the carnocyclin A; CclI, a protein with a high degree of homology to bacteriocin immunity proteins; CclC, a member of the DUF95 protein family, which was recently established to aid with immunity and transport of the mature cyclized protein (Mu et al., 2014); and CclB, CclT, and CclD, three proteins that contain membrane spanning domains, but whose functions are poorly understood (van Belkum et al., 2010). These proteins are presumed to facilitate the removal of the leader sequence, circularization, and export of the circular protein.

The post-translational maturation and export of carnocyclin A and other circular bacteriocins is poorly understood. Recent studies of garvicin ML have indicated that the cleavage of the leader peptide and cyclization of the protein occur as separate, independent events (Gabrielsen, Brede, Salehian, Nes, & Diep, 2014), but little is known about the proteins and mechanisms involved. Gabrielsen *et al*. (2014) suggested a model that circular bacteriocins are exported as linear peptides and cyclized concomitantly, but more evidence is needed to confirm this model.

Because of the large number of genes involved in the carnocyclin A production pathway characterized in *C. malteromaticum* UAL307, and the relatively poor understanding of the entire process, incorporation of this system into a non-native host may not be feasible. Optimized co-expression of the six proteins required for carnocyclin A production in a non-native host would be very difficult to achieve, especially if overexpression of the protein is desired. Furthermore, since the mechanisms involved in cyclization of carnocyclin A are poorly characterized, there is no guarantee that expression of these six proteins would result in the desired cyclical product. Thus, other cyclization systems that can be applied to the cyclic carnocyclin A need to be explored for production of this bacteriocin to be industrially feasible.

Several methodologies have been developed to produce cyclic proteins. In one method, spontaneous cyclization occurred when an N-terminal cysteine residue generated by specific proteolytic cleavage reacted with a C-terminal thioester created by an intein-mediated N to S acyl shift (Camarero & Muir, 1999). This technique has been used to produce cyclized proteins that are not normally cyclized (Camarero & Muir, 1999), as well as cyclotides (Kimura, Tran, & Camarero, 2006), which are cyclized peptides in a head to tail orientation that have high disulphide content. One limitation of this methodology is that both N-terminal and C-terminal cysteine residues are required for cyclization to occur; carnocyclin does not contain any cysteine residues.

Sortase mediated trans-peptidation has also been used to cyclize peptides: active N- and C-terminal thioesters are generated by cleavage with a site-specific sortase, Sortase A from *Staphylococcus aureus*, and the reaction proceeds similarly to the protein ligation reaction described in the previous paragraph (Parthasarathy, Subramanian, & Boder, 2007). This method has a more stringent structural requirement because it requires a sortase recognition site (LPXTG followed by several hydrophobic and then basic residues (Navarre & Schneewind, 1994). Because of the stringent structural requirements, it is therefore less appealing for use to express carnocyclin.

Another option for expression of carnocyclin is split intein circular ligation of peptides and proteins (SICLOPPS), a methodology that uses a split and permuted intein from the *Synechocystis* sp. DnaE (Figure 3-1). In this methodology, a fusion protein is created with the target protein flanked by the split and permuted intein. The intein domains interact to create an active intein that first catalyzes an N to S acyl shift at residue +1 from the C-terminus, which then undergoes transesterification to create a lariat. The lariat generates the cyclic product through asparagine side-chain cyclization. This methodology has been used to produce a number of cyclical peptides and proteins ranging from 8 amino acids to 23 kDa in length (C. P. Scott, Abel-Santos, Wall, Wahnon, & Benkovic, 1999) as well as libraries of short cyclical peptides (C. P. Scott et al., 1999; C. Scott, Abel-Santos, Jones, & Benkovic, 2001). The only structural requirement for SICLOPPS is an N-terminal cysteine, threonine, or serine residue on the residue +1 to the C-terminus of the C terminal split intein (C. Scott et al., 2001).

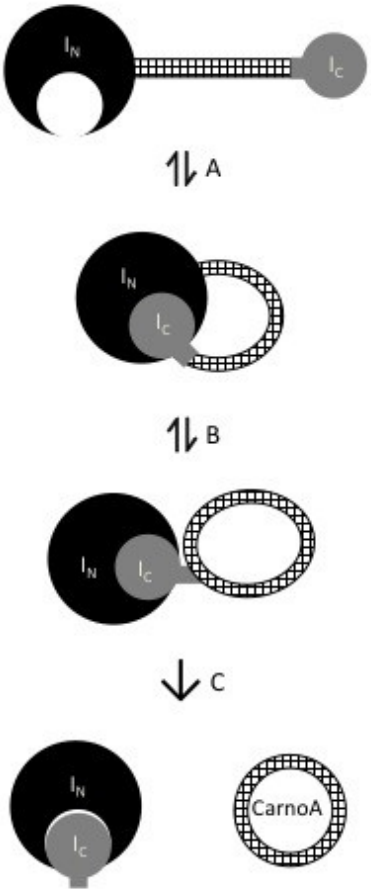


Figure 3-1 SICLOPPS methodology. A fusion protein containing the C terminal domain (I_c) and the N terminal domain (I_N) of the *Synechocystis* sp. DnaE intein flanking the protein sequence to be cyclized [in this case, Carnocyclin A (CarnoA)] is expressed. The I_N and I_C domains form a complex and catalyze an N to S (or N to O) acyl shift at the CarnoA residue +1 from I_C (Step A above), and then the intermediate undergoes transesterification to create a lariat intermediate (Step B above). The cyclic product is released from the lariat through a reaction with an asparagine side chain residue to form the products: the I_N and I_C domains and the cyclized product.

The goal of this study was to use the SICLOPPS method to produce cyclized carnocyclin in *E. coli*. The gene for the carnocyclin protein (with leader sequence removed) was cloned between the I_C and I_N sequences of the *Synechocystis* sp. DnaE with an N-terminal 6-His tag. The gene was expressed in *E. coli.* The N terminal 6-His tag was added to aid in the separation of first the full length fusion protein from the cell lysate, and then the cyclic protein from the I_C-I_N complex.

3.2 Materials and Methods

3.2.1 Strain and culture maintenance

The plasmids and strains used in this study are listed in Tables 3-1 and 3-2, respectively. Strains were grown in Luria-Bertani (LB) Broth (Thermo Fisher, Waltham, MA, USA) with appropriate antibiotics when required for plasmid maintenance. Strains were stored at -80°C in 20% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA).

Table 3-1 Plasmids used in this study

Plasmid	**Characteristics**	**Source**
pET-11d	T7 Promoter with lac Operator RBS, NcoI cloning site, T7 gene 10 leader, *Bam*HI cloning site, T7 Terminator lacIq repressor ORF	Novagen (EMD Millipore, Darmstadt, DE)
pCarnoA	pET-11d containing split intein-carnocyclin A fusion protein	Present study
pCarnoA-N-6His	pET-11d containing split intein-carnocyclin A fusion protein with an N-terminal 6His tag	Present study

Table 3-2 Strains used in this study

Organism	Characteristics	Source
Escherichia coli DH5α	Subcloning strain	Life Technologies (Carlsbad, CA, USA)
Escherichia coli BL21DE3	Expression strain	New England Biolabs (Ipswich, MA, USA)
Carnobacterium divergens NCIMB 702855	Leucocin A sensitive	(Worobo et al., 1995)
BL21pCarnoA	Expression strain carrying pCarnoA for expression of split intein-carnocyclin A fusion protein	Present study
BL21pCarnoA-N-6His	Expression strain carrying pCarnoA-N-6His for expression of split intein-carnocyclin A fusion protein with an N-terminal 6His tag	Present study

3.2.2 Cloning of the fusion protein

DNA encoding the split intein-carnocyclin A fusion protein was synthesized and codon optimized for expression in *E. coli* by Sigma-Aldrich. Genetic manipulations described below were performed using *E. coli* DH5α cells (Life Technologies, Carlsbad, CA, USA). Restriction digests were performed with enzymes also from Life Technologies. Ligations were performed using the Thermo Scientific Rapid Ligation Kit (Thermo Scientific, Pittsburgh, PA, USA). All primers were supplied by Integrated DNA Technologies (Coralville, IA, USA; sequences provided in Appendix A). Subcloning steps were performed using standard methodology (Sambrook & Russell, 2001). Product sequences were confirmed following each cloning step by Sanger sequencing using the appropriate primers and the BigDye® Terminator Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and sequenced on the ABI 3730 Sequencer (Life Technologies).

The sequence for the split intein-carnocyclin A fusion protein was amplified from the DNA template synthesized by Sigma-Aldrich with or without the addition of an N-terminal 6His tag (located on the N-terminus of the Ic domain of the split intein). Primer and template sequences are available in Table 3-3 and Appendix A, respectively. The inserts were cloned into

the *Nco*I and *Bam*HI sites of pET-11d and the desired products confirmed through sequence analysis. These plasmids were then transformed into *E. coli* BL21 DE3 cells and plated on LB solid agar supplemented with carbenicillin (Sigma-Aldrich).

Table 3-3 PCR primers used in this study.

Gene product		Primer sequence (5' to 3')
Split intein carnocyclin A	N	CCATGGTTAAAGTGATTGGTCG
	C	CATGATCCTTATCATTTAATGGTGCC
6His-tagged split intein carnocyclin A	N	CCTCCATCCCTCATCATCACCACCATCACGTTAAAG TGATT
	C	CATGATCCTTATCATTTAATGGTGCC

3.2.3 Confirming expression of the fusion proteins

Test tubes containing 5 mL of LB broth and carbenicillin were inoculated (50 µL) with an overnight culture of BL21pCarnoA, BL21pCarnoA-N-6His, or BL21pCarnoA-C-6His and shaken at 200 rpm at 37°C. When the OD_{600} reached 0.6, the cultures were induced with 1 mM IPTG and the temperature was reduced to 25°C. 0.5 mL samples were taken at 0, 3, and 20 h (after induction), pelleted, and resuspended in 50 µL (for 0 and 3 h) or 100 µL (for 20 h) of SDS load dye (100 mM Tris pH 6.8 (Sigma-Adrich), 4% SDS (Sigma-Aldrich), 20% Glycerol (Fisher), 0.2% Bromophenol Blue (Sigma-Aldrich), 10% 2-mercaptoethanol (Sigma-Aldrich)). 20 µL of the resusupended pellets were separated on a 15% SDS-polyacrylamide gel, which was subsequently stained with Coomassie Brilliant Blue for visualization of proteins.

Bands of the expected size (23 kDa) were excised from the gel and submitted for in-gel tryptic digest/LC-MS for identification at the Institute for Biomolecular Design (Department of Biochemistry, University of Alberta, Edmonton, AB, Canada).

3.2.4 Bench scale expression of the 6His-tagged split intein-carnocyclin A fusion protein

For the results described in Section 3.3, 250 mL LB and carbenicillin in a 500 mL shake flask was inoculated with 2.5 mL of an overnight culture of BL21pCarnoA-N-6His and shaken at 200 rpm and 37°C. When the OD_{600} reached 0.6 (3 h), protein expression was induced with 1 mM IPTG. The culture was shaken at 200 rpm and 25°C for 3 h, and then the culture was centrifuged (4 000 x *g*, 15 min) and the pellet was flash frozen to -80°C and then stored at -20°C.

3.2.5 Affinity purification of the 6His-tagged split intein-carnocyclin A fusion protein

For the results described in Section 3.3, the following protocol was followed: the frozen cell pellet from section 3.2.4 was resuspended in 5 mL cyclization buffer (20 mM Tris (Sigma-Aldrich), 1 mM TCEP (Sigma-Aldrich), and 0.5 mM sodium chloride (Fisher), pH 7.8) (Tavassoli & Benkovic, 2007). The resuspended pellet was sonicated six times for 20 s, and then centrifuged at 17 000 x *g* for 10 min. The supernatant was loaded onto a 5 mL Ni-NTA (Life Technologies) resin, incubated for 10 min at room temperature, and the column was washed once with cyclization buffer. The column was then incubated at 25°C overnight without agitation to allow for cyclization. Following the overnight incubation, the column was washed three times (by gravity flow) with 5 mL of cyclization buffer, followed by three elutions with cyclization buffer containing 250 mM imidazole, pH 7.8 (Sigma-Aldrich).

3.2.6 Alternate expression and purification methodologies

In addition to the protocols described in sections 3.2.4 and 3.2.5, induction temperatures were varied (25°C, 30°C, and 37°C), as well as induction length (varied from 2 h to overnight). In addition, protease inhibitors (PMSF (Sigma-Aldrich), 1 mM and Pepstatin (0.01 mM (Sigma-Aldrich)), were added to the protein lysis solution.

3.2.7 Analysis for expression, cyclization progress, and activity of carnocyclin A

All wash and elution fractions from section 3.2.5 were analyzed using a spot on lawn assay for bacteriocin activity and SDS-PAGE. For the spot on lawn assay, a petri dish containing APT (Difco™) agar (Fisher) was overlaid with 5 mL of APT soft agar (0.75% agar (w/v)) inoculated with 50 µL of an overnight culture of *Carnobacterium divergens* NCIMB 702855. The agar was allowed to solidify, and then 10 µL of the test solution was spotted on the plate.

The plate was allowed to dry under a biosafety cabinet for approximately 15 min, and then the plate was incubated overnight at 25°C. For the SDS-PAGE analysis, 20 μL of each fraction was mixed with load dye and loaded on a 15% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue.

3.2.8 Incubation in conditions to support cyclization

Concentrated elution fractions were incubated with 0.1 mM, 1 mM, or 10 mM DTT, 20 mM Tris, (pH 7.6), and 0.5 mM NaCl at 35°C, 55°C, or 75°C for 4 h. The concentration of DTT and incubation times were varied in an attempt to optimize/promote cyclization. Samples were subjected to a spot on lawn assay or SDS-PAGE analysis as described in Section 3.2.6.

3.2.9 MALDI-MS

Samples were submitted to the Mass Spectrometry Facility (Department of Chemistry, University of Alberta) and analyzed on a Bruker Ultraflextreme MALDI TOF/TOF (Bruker Daltonics, Bremen, GmBH), with a sinapinic acid matrix.

3.3 Results

3.3.1 Confirmation of expression

The coding sequence for the split intein-carnocyclin A fusion protein with and without an N-terminal 6His tag were cloned into pET-11d to create constructs for SICLOPPS (Figure 3-2). The resulting plasmids were sequenced to confirm the identity of the desired plasmid and to verify that mutations were not present. For simplicity, the following sections do not mention the existence of the split intein in descriptions of the protein or expression strains as this element is present in all plasmids and proteins containing carnocyclin A.

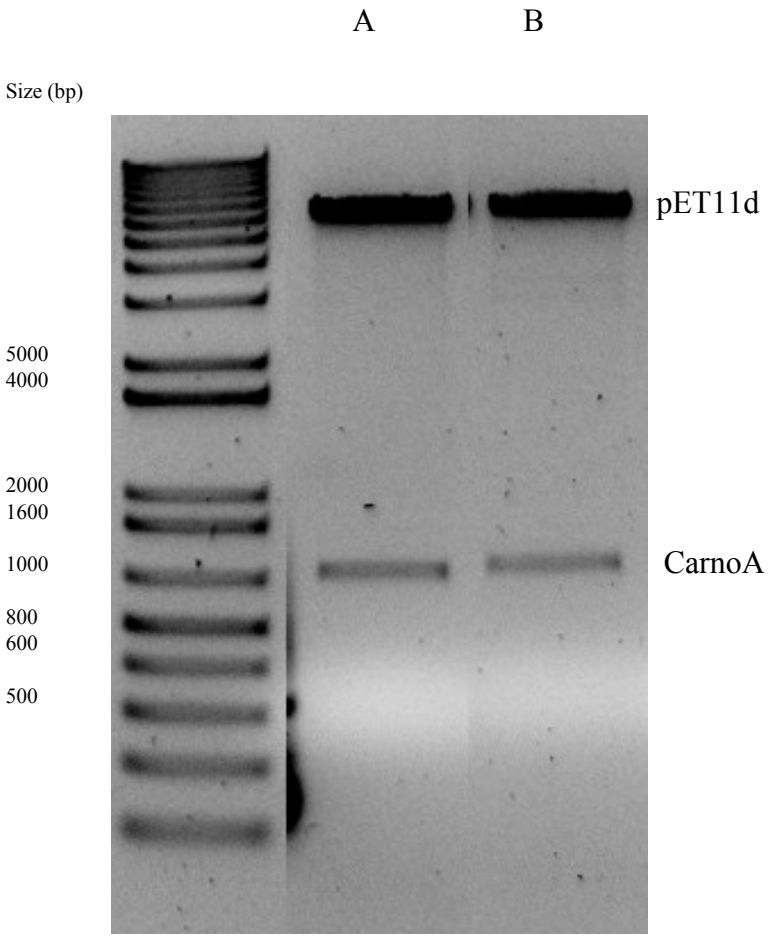


Figure 3-2. Cloning gel showing carnocyclin A clones, Carno (A), and 6His-Carno (B)

Expression of the untagged and 6His-tagged carnocyclin A was performed in *E. coli* BL21DE3 cells and the cell pellets were analyzed by SDS-PAGE (Figure 3-3). Bands of the expected molecular weights (23.0 kDa and 23.8 kDa for the untagged and 6His-tagged protein, respectively) appeared after 2 h of induction and remained after 20 h. The 2 h band was excised from the gel and subjected to tryptic digestion and LC-MS. Fragments matching the sequence of the desired proteins are presented in Table 3-4 and 3-5.

For the fusion protein without the 6His tag, the fragments cover the sequence of the protein from residue V89 to the C-terminal lysine residue (K213). Although full coverage was

not obtained, these data combined with the apparent molecular weight observed in SDS-PAGE suggest that the full-length carnocyclin A protein is being expressed. In addition, sequence analysis of the expression vector (data not shown) indicates that the clone was not truncated at the N terminus.

For the 6His-tagged fusion protein, complete sequence coverage of the protein was obtained through tryptic digestion and LC-MS with the exception of residues 15-20, 68-78, 96-116, and 116-169 (Table 3-4 and Table 3-5). The apparent molecular weight for the 6His-tagged Carnocyclin A is well above 25.0 kDa (Figure 3-3). This is higher than expected (23.8 kDa), but can be explained by the positive charge imparted on the protein by the 6His tag. The combination of plasmid sequencing, the observed molecular weight, and the sequence coverage obtained by tryptic digestion and LC-MS suggest that the 6His-tagged carnocyclin A is also being expressed successfully.

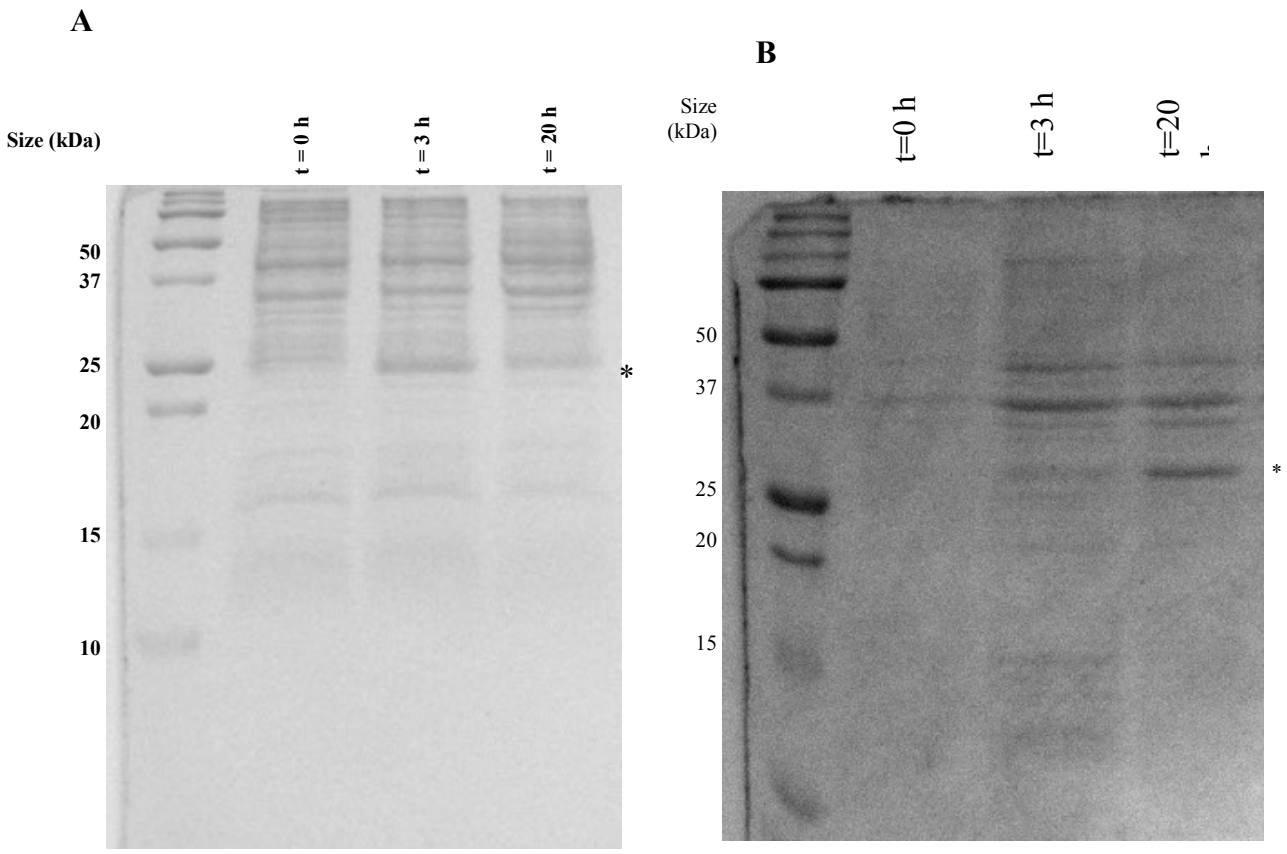


Figure 3-3 SDS-PAGE gel of split intein-carnocyclin A expression **(A)** and 6His-carnocyclin **(B)** before and after induction. The asterisk indicates the size of the band excised for tryptic digestion and LC-MS.

Table 3-4 Peptide fragments from tryptic digestion and LC-MS and their respective sequence coverage of carnocyclin A

Peptide Fragment Sequence	**Coverage**
QGIKKAIQLLVAYGIAQGTAEK	Q67-K88
KAIQLLVAYGIAQGTAEK	K71-K88
AIQLLVAYGIAQGTAEK	A72-K88
VVcLSFGTEILTVEYGPLPIGK	V89-K110
IVSEEINcSVYSVDPEGR	I111-R128
VYTQAIAQWHDRGEQEVLEYELEDGSVIR	V129-R157
VYTQAIAQWHDR	V130-R140
GEQEVLEYELEDGSVIR	G142-R157
ATSDHRFLTTDYQLLAIEEIFARQLDLLTLENIK	A158-K191
FLTTDYQLLAIEEIFAR	F164-R180
QLDLLTLENIK	Q181-K191
QLDLLTLENIKQTEEALDNHR	Q181-R201
QLDLLTLENIKQTEEALDNHRLPFPLLDAGTIK	Q181-K213
LPFPLLDAGTIK	L203-K213

Table 3-5 Peptide fragments from tryptic digestion and LC-MS and their respective sequence coverage of 6His-tagged carnocyclin A

Peptide Fragment Sequence	Coverage
MHHHHHHVKVIGRR	M1-R14
IFDIGLPQDHNFLLANSLINAGLTVGSIISILGGVTVGLSGVFTAVK	I21-K67
KAIqLLVAYGIAQGTAEK	K77-K94
AIQLLVAYGIAQGTAEK	A78-K94
KAIQLLVAYGIAQGTAEK	K78-K94
AIqLLVAYGIAqGTAEK	A78-K95
IVSEEIncSVYSVDPEGR	I117-R134
VYTqAIAQWHDRGEQEVLEYELEDGSVIR	V135-R163
GEQEVLEYELEDGSVIR	G147-R163
FLTTDYQLLAIEEIFAR	F170-R186
QLDLLTLEnIKQTEEALDNHR	Q187-R207
QLDLLTLENIKQTEEALDNHRLPFPLLDAGTIK	Q187-K219
qLDLLTLEnIKqTEEALDnHR	Q187-R207
QLDLLTLENIK	Q188-K197
QLDLLTLENIKQTEEALDNHR	Q188-R207
qTEEALDnHRLPFPLLDAGTIK	Q198-K219
LPFPLLDAGTIK	L208-K219

3.3.2 Attempts to increase expression and promote cyclization

After production of the full-length protein was confirmed for both the untagged and 6His-tagged carnocyclin A, scale-up production of the latter was pursued in order to facilitate cyclization and purification of the protein. The crude cell lysate was applied to a Ni-NTA column, washed, and incubated overnight to allow for cyclization. The column was then washed, and the protein of interest was eluted using a buffer containing 250 mM imidazole. The washes were separated on an SDS-polyacrylamide gel (Figure 3-4) and a spot-on-lawn assay was performed (Figure 3-5).

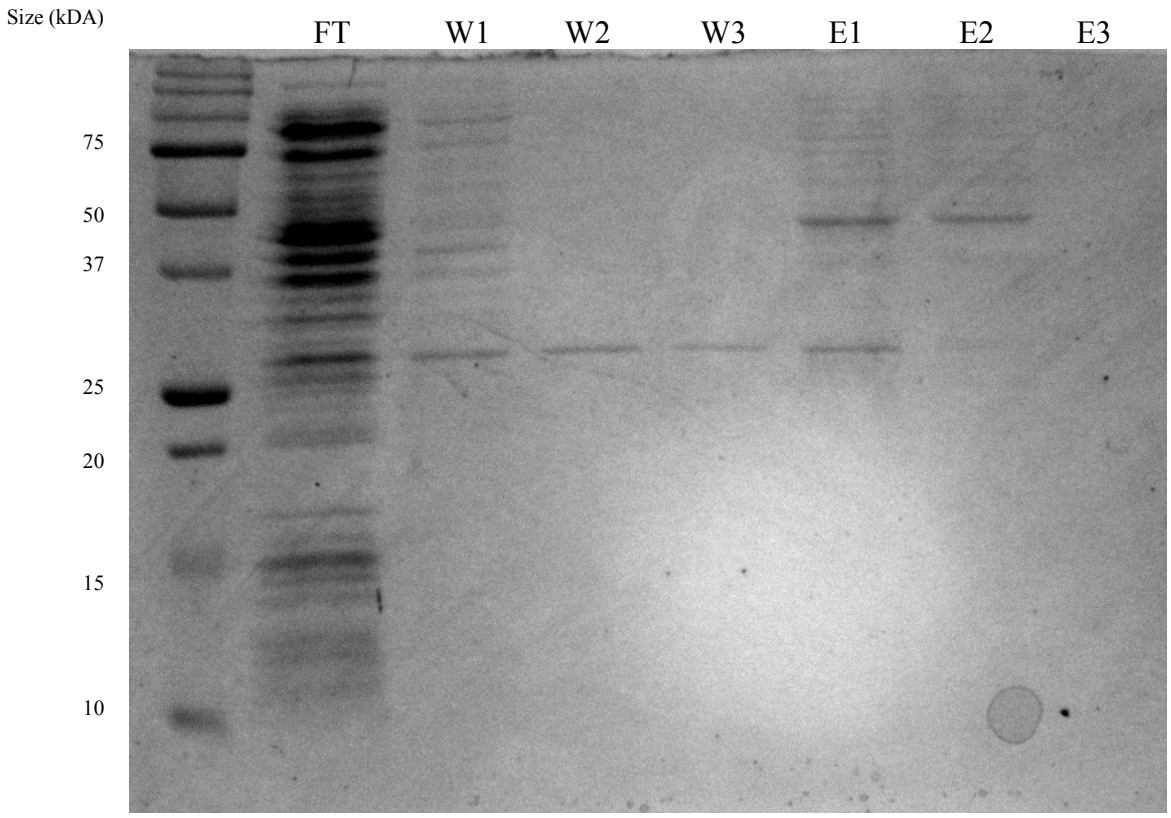


Figure 3-4 SDS-PAGE of 6His-tagged carnocyclin A purification. Flow-through obtained following overnight incubation (FT). Washes (W1, W2, W3) and elutions (E1, E2, E3) were captured from a Ni-NTA column.

Several attempts were made to increase the expression and purification yields for the fusion protein. As described in Section 3.2.6, induction temperature and length of induction time were varied, and protease inhibitors were added in attempts to increase protein yield. None of these variations produced a significantly increased yield of the fusion protein when assayed by SDS-PAGE.

The expected molecular weights for the full-length linear protein, cyclization intermediate, and final cyclization product are indicated in Table 3-5. Since the 6His tag is present at the N-terminus of the protein, during the cyclization process, the 6His tag is removed from the carnocyclin A product. Thus, the cyclized protein was expected in the 0 mM imidazole wash, while the unprocessed carnocyclin A, the lariat intermediate, and the 6His-tagged intein complex were expected to elute in the 250 mM imidazole wash. The two intein fragments were

expected to interact under the non-denaturing conditions of the experiment and should thus co-elute.

Neither the cyclization intermediate nor the products were visible in the 0 mM imidazole wash, although several of the products are too small to be visualized on this SDS-PAGE gel (Table 3-6) and the Coomassie stain used may not be sensitive enough for small amounts of protein. The full-length protein appears in both the 0 mM imidazole wash and the 250 mM imidazole wash, indicating that there may be poor interaction between the 6His tag and the column. The spot-on-lawn assay (Figure 3-5) indicates that there is no bacteriocin activity in any of the wash or elution fractions. Despite the discoloured appearance in the image, the dark spot in the flow-through fraction is not a zone of clearing. The lack of activity indicates that the protein is not undergoing cyclization as expected.

Table 3-6 Expected bands from 6His-tagged carnocylin A cyclization

Peptide	Description	Expected molecular weight (kDa)
His-Ic-Carnocyclin-In	Full length protein	23.8
His-Ic-Carnocyclin	Lariat intermediate	10.0
His-Ic	Product	4.2
Carnocyclin	Product	5.8
In	Product	13.9

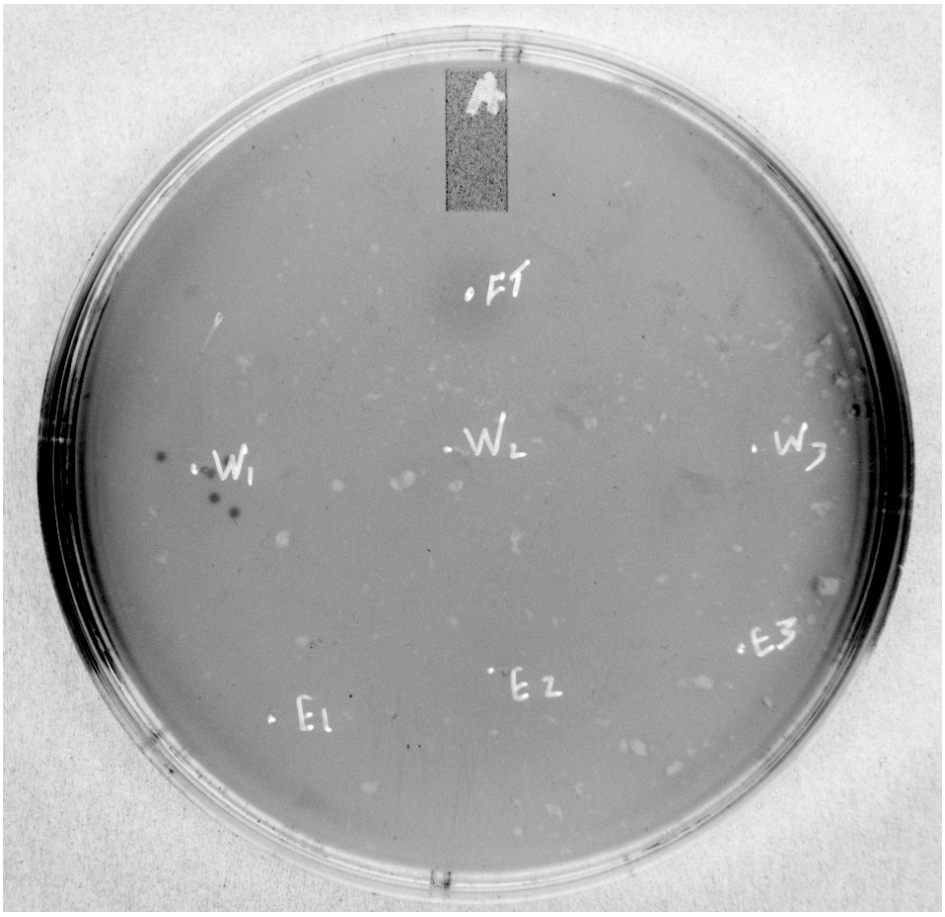


Figure 3-5 Spot on lawn assay of flow-through (FT), wash (W1, W2, W3), and elution (E1, E2, E3) fractions from the Ni-NTA column.

The results indicate that the full-length 6His-tagged carnocyclin A protein is not undergoing cyclization on the Ni-NTA resin through the mechanism described in Figure 3-1, or not going through enough cyclization to be detected. With the intention of promoting cyclization through this mechanism, the full-length carnocyclin A (with the split intein) eluted from a Ni-NTA column was incubated in cyclization buffer with 1, 10, or 100 mM DTT at 25°C, 45°C, or 65°C. Although TCEP was used in the cyclization buffer, varying concentrations of DTT were added to these reactions in order to maintain a reducing environment during mixing in this experiment. Different temperature incubations were performed in an attempt to promote cyclization because *Synechocystis sp.* generally have optimal growth between 30°C and 45°C

(Sheng et al., 2011), and the optimal protein cyclization temperature may reflect this optimal growth temperature. The protein was then separated on an SDS-polyacrylamide gel (Figure 3-6) and a spot on lawn assay (Figure 3-7) was performed. Increasing temperature or concentration of DTT promoted fragmentation or decomposition of the protein (Figure 3-6). However, the spot on lawn assay indicates that the protein is not being processed into the active cyclic peptide. To further understand the mechanism of fragmentation, MALDI-MS was performed on the fragmented samples. None of the fragments from MALDI-MS match the fragmentation pattern expected if the reaction was proceeding through cyclization (data not shown).

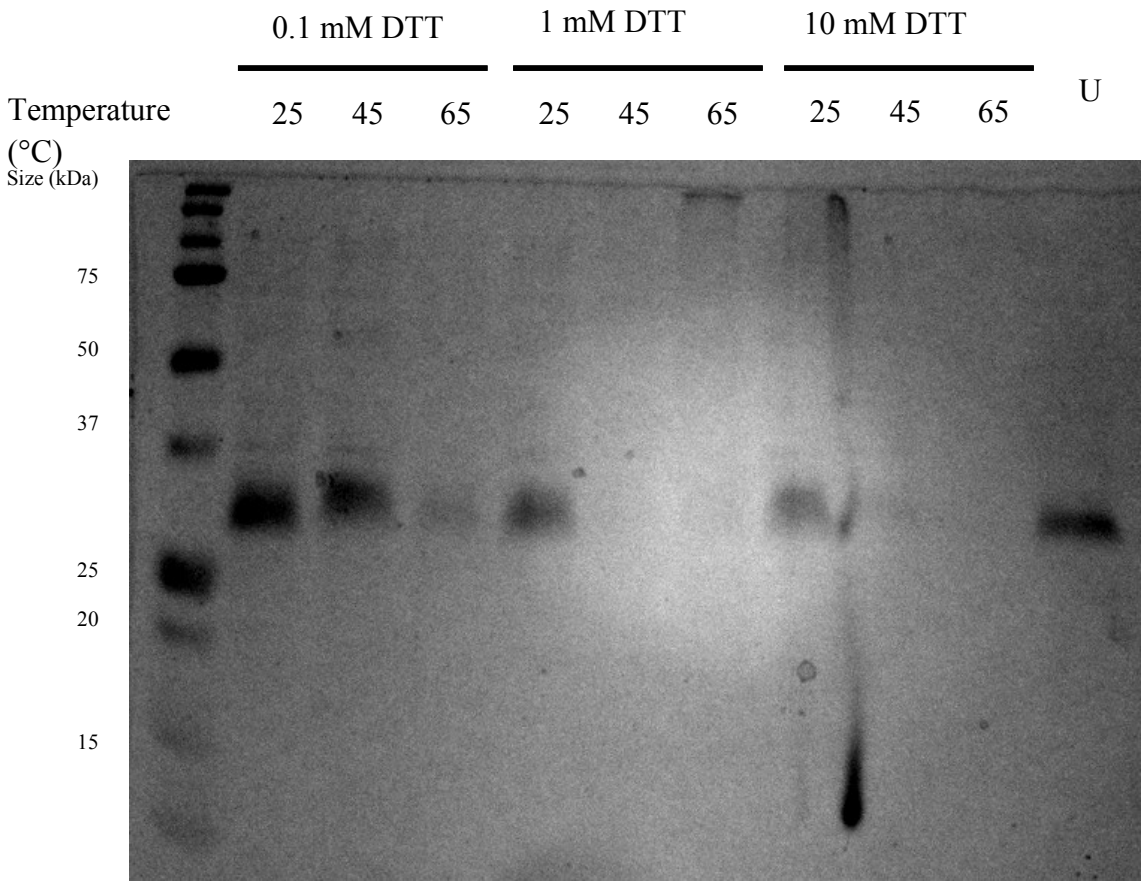


Figure 3-6 SDS PAGE of carnocyclin A from Ni-NTA column elution after incubation with 0.1, 1, or 10 mM DTT at 25°C, 45°C, or 65°C for 4 h. Lane U contains untreated sample.

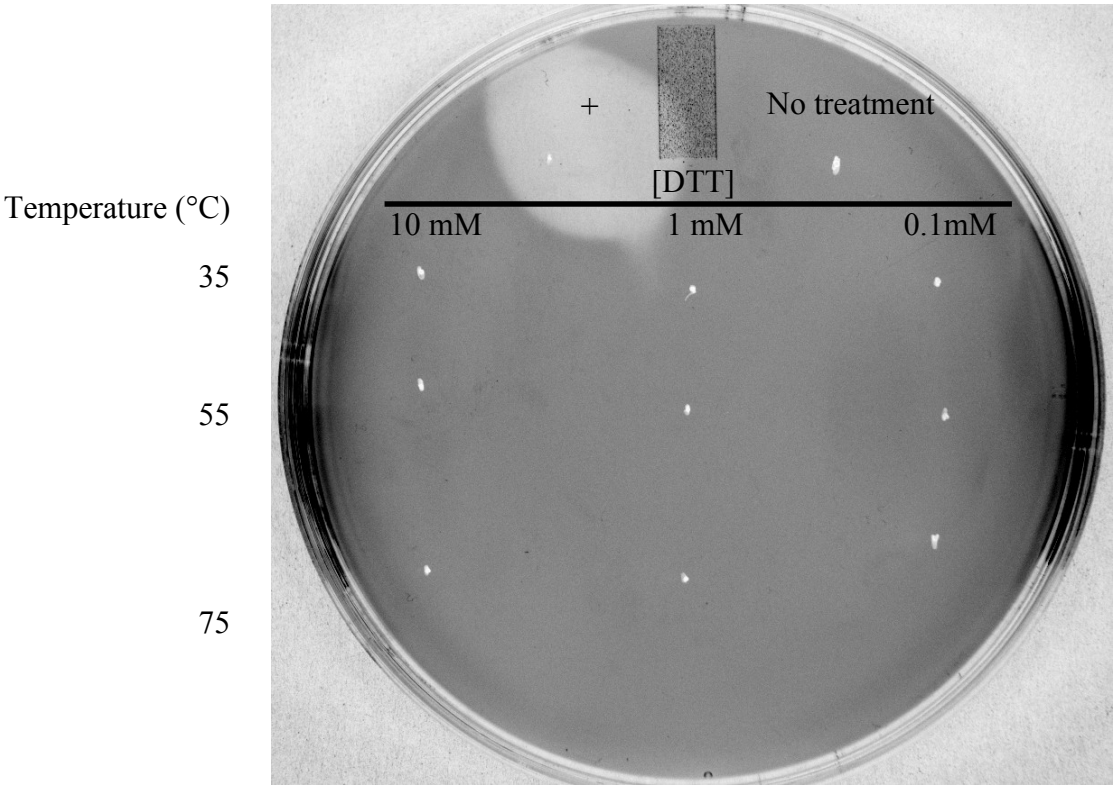


Figure 3-7 Spot on lawn activity assay for carnocyclin A from Ni-NTA column elution after incubation with 1, 10, or 100 mM DTT at 35°C, 55°C, or 75°C for 4 h. Positive control (leucocin A) indicated by +, negative control (untreated elution) indicated by "no treatment".

3.4 Discussion

We have successfully expressed the untagged and 6His-tagged split intein carnocyclin A protein fusion in *E. coli* and confirmed expression by tryptic digestion and LC-MS. The use of the SICLOPPS system to cyclize this protein is promising, though we have not yet successfully cyclized carnocyclin A using this methodology. When Deschuyteneer *et al*. (2010) used a similar technology to produce a library of randomized peptides, they found that approximately 50% of the random peptides were ligated. It is important to note that this is for a library of random peptides and therefore random peptide structures; the physical proximity of the N and C termini of carnocyclin A should improve the probability of the reaction, although the chosen breakpoint

in the carnocyclin A structure in this study may interfere with proper folding. There are several possible reasons why cyclization has not occurred: the mostly likely explanations are discussed below.

The size of the peptide or protein to be cyclized can have an effect on success of the SCILOPPS technique. A peptide that is too small could constrain the cyclization system by preventing the split intein from forming a complex because the molecule will not be able to bend enough, while a peptide that is too large could have large tertiary structure that prevents the split intein domains from interacting. SICLOPPS has been previously applied to produce functional cyclized peptides and proteins ranging in size from 8 amino acids to 23 kDa in the same strain of *E. coli* used in this study (C. P. Scott et al., 1999). Carnocyclin A, a 5.9 kDa protein, is within the size range of previous expressed proteins, so the size of the protein is not likely physically preventing the cyclization.

The native three-dimensional proximity of the N- and C-termini of the protein being cyclized could play a role in the efficiency of the cyclization. Based on the crystal structure of the protein, the serine residue chosen as the N-terminal residue of carnocyclin A is within an alpha helix (Martin-Visscher et al., 2009). The chances of cyclization may be improved if the break in the protein structure is at a serine residue that is not within a secondary structural element because this could allow for improved folding of carnocyclin A, bringing the N- and C-termini closer together in three dimensional space. There is one serine residue within the structure of carnocyclin A that is at the beginning of an alpha helix that may be a better choice. This residue (GS) is highlighted in Appendix B.

A study of protein synthesis by native chemical ligation of peptides, which undergo similar chemistry to the SICLOPPS reaction, indicated that the C-terminal residue plays a role in the rate of cyclization (Hackeng, Griffin, & Dawson, 1999). When alanine and glycine were the C-terminal residues, the reaction had the fastest ligation rates, while C-terminal β-branched amino acids like leucine, valine, threonine, and isoleucine had slower ligation rates, although the peptides containing the β-branched amino acids still achieved some ligation (Hackeng et al., 1999). Scott *et al.* (2001) were also able to ligate peptides using the SICLOPPS method when an isoleucine residue was in the C-terminal position. In this experiment, the C-terminal amino acid was valine; it is possible that the C-terminal valine may be slowing the rate of ligation. In future

experimental work on this project, cyclization may be achieved by choosing a different serine residue as the N-terminal residue, specifically the serine residue that follows the glycine residue (highlighted in Figure B-1, Appendix B), which would create a peptide where the C-terminal residue is a glycine. Alternately, an expression of a different Class IIc bacteriocin using a similar system could be attempted.

One major deviation from the methodology used by Tavassoli and Benkovic (2007) is the use of an N-terminal 6His affinity tag to isolate the full-length protein, rather than a C-terminal tag. The N-terminal 6His tag may inhibit the cyclization reaction by structurally preventing the two domains of the split intein from interacting. The charge imparted by the His residues of the tag may also alter the solubility and structure of the C-terminal component of the split intein (the N-terminus of the construct used in this study). This could cause unwanted intra or intermolecular interactions that could be preventing the interaction of the split intein fragments. A C-terminal His-tagged carnocyclin A was cloned in this study, however, expression was not achieved. A comparison of protein crystal structures with and without His tags indicated that His tags generally do not impact protein structures (Carson, Johnson, McDonald, Brouillette, & DeLucas, 2007). However, several groups have recently reported that His tags affect the protein activity of specific proteins (Charbonneau, Meddeb-Mouelhi, & Beauregard, 2012; Dickson, Lee, Shepherd, & Buchanan, 2013; Wu et al., 2012). There are no reports on the effect of the chitin binding domain on protein structure or functions, but this does not mean that chitin binding domains do not interfere with protein structure or function, and could theoretically interfere as much as or more than a His tag. The effect of these purification tags on structure and activity needs to be considered on a case-by-case basis.

Although we achieved expression of the protein in this experiment, it is possible that expression may be improved. Induction time, temperature, and the concentration of IPTG could be fully optimized. Different expression strains or systems should also be explored. Presuming that cyclization of this protein can be achieved, optimization of expression would help generate an industrially-relevant production system.

Chapter 4: Combinations of antimicrobials for use against industrial spoilage organisms

4.1 Introduction

As discussed in detail in Chapter 1, a number of antimicrobials have been studied for use against industrial spoilage organisms, including conventional antibiotics (Hynes et al., 1997), hop extracts (Ruckle & Senn, 2006), and bacteriocins (Peng et al., 2012); all of these antimicrobials have varying degrees of effectiveness against industrial spoilage strains. This varying degree of effectiveness can potentially cause overdosing or underdosing of antimicrobials when trying to control spoilage. This is problematic because overdosing unnecessarily adds to the cost of production while underdosing isn't effective against the contamination and can lead to the development of antimicrobial resistant strains (Rammelkamp & Maxon, 1942). Strains of *Lactobacillus*, the genus primarily responsible for contamination in an industrial context, exhibit varying degrees of sensitivity to each of the inhibitors used in this study (Breuer & Radler, 1996; Rich et al., 2011; Simpson & Fernandez, 1994).

Industrial ethanol fermentations, like many food processing systems, may be concurrently contaminated with a number of different strains with varying sensitivities to antimicrobials (Lucena et al., 2010) and mixed spoilage cultures of two or more contaminating species are often observed in industrial ethanol production (Lucena et al., 2010). Combining antimicrobials can be useful to overcome the challenges associated with the presence of strains with varying degrees of sensitivity in a mixed culture scenario. An example that is commonly applied in the food industry is the use of antimicrobial hurdle technologies, where two or more antimicrobial "hurdles" at relatively mild doses are added to the system (Leistner & Gorris, 1995). This hurdle technology may also be applied to help control strains associated with spoilage in industrial bioethanol production.

When inhibitors are combined, there is also potential for antimicrobials to exhibit antagonistic, additive, or synergistic properties. A synergistic property occurs when the combined effect of the antimicrobials is greater than the effect of each antimicrobial alone (Denyer, Hugo, & Harding, 1985). Synergy between antimicrobials can drastically lower the required dose, providing a major cost savings. Synergy between antimicrobials occurs when the antimicrobials have mechanisms of action that are unique from one another, but are related in a

way that allows their combined effect to be greater than the sum of each inhibitor on its own (Denyer et al., 1985). A synergistic antimicrobial combination would offer great cost savings to industry and is highly desired.

In this study, the antimicrobials nisin, Isostab® (a hop extract), Lactrol (active ingredient is virginiamycin), and leucocin A were studied alone and in combination against a panel of industrial spoilage organisms. Each of these antimicrobials have unique mechanisms of action, although some of them are related. Nisin and leucocin A are both bacteriocins and share a general mechanism of action of permeabilizing the cell membrane and disrupting the proton motive force (Bruno & Montville, 1993). However, these bacteriocins rely on different receptors for membrane binding. Nisin binds to the cell wall precursor lipid II (Brotz et al., 1998), while class IIa bacteriocins, including leucocin A, are believed to bind to the cell mannose phosphotransferase system (D. B. Diep, Skaugen, Salehian, Holo, & Nes, 2007). Hop compounds also work by dissipating the proton motive force, but do so by imbedding in the cell membrane and acting as ionophores (Simpson, 1993). Lactrol interferes with cellular protein synthesis by preventing interactions between the ribosome and elongation factors (Chinali et al., 1981). The four antimicrobials were evaluated for synergistic properties and the potential for various combinations to benefit industrial ethanol producers.

4.2 Materials and Methods

4.2.1 Culture maintenance

The strains used in this study are listed in Table 4-1. Strains were stored at -80 °C in 20% (v/v) glycerol in Difco™ All Purpose Tween (APT) media (Franklin Lakes, NJ).

Beer spoilage strains and *Leuconostoc gelidum* UAL187 were grown in APT broth at 25 °C without shaking. Culturing of *L. gelidum* UAL187 in a bioreactor is described in section 4.2.2 below.

Table 4-1 Strains used in this study, their characteristics, and their sources

Strain	Characteristics	Source
Lactobacillus brevis N104	beer spoilage	(Yansanjav et al., 2004)
Lactobacillus brevis G433	beer spoilage	(Yansanjav et al., 2004)
Lactobacillus plantarum G326	beer spoilage	(Yansanjav et al., 2004)
Lactobacillus buchneri N214	beer spoilage	(Yansanjav et al., 2004)
Lactococcus lactis MG1363	genetically characterized	(Gasson, 1983)
Leuconistoc gelidum UAL 187	leucocin A producer	(Hastings & Stiles, 1991)
Carnobacterium divergens NCIMB 702855	leucocin A sensitive	(Worobo et al., 1995)

4.2.2 Production and purification of Leucocin A

Production and purification of Leucocin A was performed as per section 2.2.4.

Arbitrary activity units (AU) were determined by performing a spot on lawn assay (see Section 4.2.3) with serial dilutions of the purified bacteriocin. Within this study, an AU is defined as the amount of activity required to give a 1.0 cm zone of clearing on an APT plate seeded with *C. divergens* LV13.

4.2.3 Spot-on-lawn assays

Spot-on-lawn assays were performed as per section 2.2.4, except the plates were incubated for 24-48 h, depending on the strain. *La. plantarum* G326 and *Lc. lactis* MG1363 were observed at 24 h, while, *La. buchneri* N214, *La. brevis* N104, *La. brevis* G433 were observed at 48 h.

4.2.4 Minimum inhibitory concentration assays

An adapted version of the Clinical Laboratory Standards Institute (CLSI) test method (Garcia, 2010a) was used. Solutions of the microbial inhibitors nisin (Sigma-Aldrich), Isostab® (BetaTech Hop Products; Washington, USA), Lactrol (PhibroChem; Teaneck, NJ, USA), and leucocin A (purified above) were diluted across a sterile 96 well, flat bottom, polystyrene plate (Fisher). Overnight cultures of beer spoilage strains were normalized to OD_{600}=0.05, then diluted 1:200 in APT media. Wells were inoculated with 90 µL of the diluted culture (initial cell density ~2.0 X 10^5 CFU/mL), sealed, and incubated at 30 °C without agitation. Following incubation for

20 h (*Lactobacillus plantarum* and *Lactococcus lactis* strains) or 44 h (*Lactobacillus brevis* and *Lactobacillus buchneri* strains), the wells were mixed by pipetting and absorbance was measured using a Synergy MX Plate Reader (BioTek, Winooski, VT) and Gen5 software (BioTek). The minimum inhibitory concentration (MIC) for each inhibitor was determined as the lowest concentration that resulted in no increase in optical density. Wells were considered to have no growth if the measured optical density was within 10% of the blank reading. The use of the OD_{600} reading was the major experimental deviation from the CLSI methodology, which suggests determination of growth/no growth by a visual examination of the culture for turbidity or the presence of a pellet at the bottom of the well. Because of concerns regarding reproducibility of the visual assay, especially when culture "trailing" was present, we chose to use the plate reader. All values reported represent the result of triplicate experiments, where the experiment was performed on three separate days using fresh antimicrobial solutions, media, and cultures. When experimental values didn't agree, the lowest MIC value was reported and used in further experiments.

4.2.5 Checkerboard experiment

An adapted version of the Clinical Laboratory Standards Institute (CLSI) test method (Garcia, 2010b) was used. Solutions of the microbial inhibitors nisin (Sigma-Aldrich), Isostab® (BetaTech Hop Products), Lactrol® (PhibroChem), or leucocin A (purified above) were diluted across or down a sterile 96 well, flat bottom, polystyrene plate (Fisher) starting with 2X the MIC (determined in section 4.2.4) for each inhibitor. This created an array of wells containing 64 different combinations of each pair of inhibitors, with concentrations ranging from 0X to 2X the MIC.

Cultures of each of the five strains were normalized to $OD_{600} = 0.05$, then diluted 1:178 (to duplicate the dilution used in the MIC assay) in 1.1X APT media. Wells of the checkerboard plates were inoculated with 80 μL of the diluted culture to give the same final inoculum cell concentration as section 4.2.4. Following inoculation, the plates were sealed with sealing tape and incubated at 30°C without agitation. Incubation of plates and determination of culture growth through absorbance measurements were performed as described for MIC in 4.2.4.

For each well displaying no growth that bordered a well with growth, the Fractional Inhibitory Concentration (FIC) of each inhibitor and the sum of the FICs (ΣFIC) were determined using the following equations:

$$\Sigma FIC = FIC_X + FIC_Y = C_X/MIC_X + C_Y/MIC_Y$$

where C_X and C_Y are the concentrations of inhibitors X and Y, respectively, when used in combination, and MIC_X and MIC_Y are the minimum inhibitory concentrations of inhibitors X and Y, respectively, when used alone. Based on this equation, expressing inhibitor concentrations as fold MIC is identical to indicating the FIC of an inhibitor. For example, if inhibitor X is present at a concentration of 2-fold MIC, then:

$$FIC_X = C_X/MIC_X = 2(MIC_X)/(MIC_X) = 2$$

The lowest (ΣFICmin) and highest (ΣFICmax) FIC sums for each combination of inhibitors were reported. The experiment was performed in triplicate on different days with fresh antimicrobial solutions, media, and cultures; the values are the lowest and highest values among the three experiments. The complete set up of the checkerboard array and determination of ΣFIC, ΣFICmin, and ΣFICmax are explained in Appendix C.

4.2.6 Shake flask experiment

Five hundred mL shake flasks containing 250 mL Difco™ APT broth and 12.5 mg/L (1X MIC) or 6.8 mg/L (0.5X MIC) nisin, and/or 50 mg/L (1X MIC) or 25 mg/L (0.5X MIC) Isostab® were inoculated with 1.0 mL of an *La. plantarum* culture that had been normalized to OD_{600} = 0.0625. The flasks were fitted with a gas trap and shaken at 200 rpm at 30°C. Samples (1mL) were taken aseptically at 0, 6, 24, 48, and 72 h and a full dilution series was plated on Plate Count Agar (PCA) (EMD Millipore, Billerica, MA, USA). Plates were counted after a 72 h incubation at 30°C. The experiment was performed in triplicate, error bars represent standard deviation.

4.3 Results

4.3.1 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of each antimicrobial for each beer spoilage strain was determined (Table 4-2). *Lactobacillus spp.* gave MIC values of 12.5-25.0 μg/mL for nisin, values that are slightly higher than, but within an order of magnitude of the 3-10 μg/mL range previously reported by groups with similar, but not identical, methodologies (M. A. Franchi et al., 2012; M. Franchi, Serra, & Cristianini, 2003; Turgis, Khanh Dang Vu, Dupont, & Lacroix, 2012). The MIC for nisin against *Lactococcus lactis* MG1363 was 0.4 μg/mL, an order of magnitude lower than that observed for *Lactobacillus* strains. However, this value is an order of magnitude higher than the 0.040 μg/mL value previously reported in the literature for this strain (Kramer, Van Hijum, Knol, Kok, & Kuipers, 2006), although a different test method was used (specifically, using a spectrophotometer rather than a visual test to determine the breakpoint in the plates between growth and no growth) which may account for the difference in reported values.

Sensitivity to Isostab® varied from 50-200 μg/mL. The *Lactobacillus brevis* and *Lactococcus lactis* strains had lower sensitivity. This range was higher than, but within an order of magnitude of, the 30-80 μg/mL range reported in a study done on the Isostab® products (Ruckle & Senn, 2006).

Lactobacillus strains were inhibited by 0.32-0.63 μg/mL of Lactrol. This value is comparable to the 0.5 μg/mL effective dose established for the inhibition of *Lactobacillus spp.* in wheat mash systems (Hynes et al., 1997). This is also below the manufacturer's recommended starting dosage of 2 μg/mL (Phibro Ethanol Performance Group, 2010). *Lactococcous lactis* MG 1363 was less sensitive to Lactrol, with a MIC value of 2.50 μg/mL.

Table 4-2 Minimum inhibitory concentrations for antimicrobials against *Lactobacillus* and *Lactococcus* strains, NI = no inhibition at highest concentration tested

Strain	Minimum Inhibitory Concentration			
	Nisin (μg/mL)	Isostab® (μg/mL)	Lactrol (μg/mL)	Leucocin A (AU/mL)
Lactobacillus brevis N104	12.5	200	0.63	NI
Lactobacillus brevis G433	25.0	100	0.63	NI
Lactobacillus plantarum G326	12.5	50	0.32	NI
Lactobacillus buchneri N214	25.0	50	0.63	NI
Lactococcus lactis MG1363	0.4	100	2.50	NI

An MIC value could not be determined for leucocin A against any of the strains studied. This is remarkable because leucocin A inhibits all of *Lactobacillus sp* used in this study based on a spot on lawn assay with the exception of *Lactobacillus brevis* N104 (Figure 4-1). Leucocin A was used at a final concentration of up to 160 AU/mL in the liquid assay, while only 16 AU was required to give a zone of clearing in spot on lawn assays.

Table 4-3 Results of spot on lawn assay of leucocin A against beer spoilage strains. + indicates a zone of clearing was observed, - indicates no zone of clearing.

Strain	Spot-on-lawn result
Lactobacillus brevis N104	-
Lactobacillus brevis G433	+
Lactobacillus plantarum G326	+
Lactobacillus buchneri N214	+
Lactococcus lactis MG1363	-

4.3.2 Checkerboard experiment

A checkerboard experiment was performed for each strain against each possible combination of inhibitors. Checkerboard experiments measure the effectiveness of each pair of inhibitors in combination; the inhibitors were combined at concentrations ranging from 0X to 2X their individual MICs. A typical plate layout for a checkerboard experiment is shown in Appendix C.

For each well showing no growth that is positioned at the breakpoint between the growth and no growth sections of the plate, the fractional contribution of each inhibitor was determined and the sum or the fractional inhibitory concentrations (ΣFIC) was calculated. In each checkerboard experiment, there were multiple wells that satisfied these requirements. Thus, the lowest (ΣFIC_{min}) and highest (ΣFIC_{max}) sums of fractional inhibitory contributions obtained from all such wells from three unique experiments are reported in Table 4-4. A further explanation of ΣFIC, ΣFIC_{min}, and ΣFIC_{max} is given in Appendix C. Because a minimum inhibitory concentration for leucocin A could not be determined, only the fractional inhibitory contribution of the non-leucocin inhibitor in the combination is reported (Table 4-5).

An ΣFIC_{min} value of >4.0 is considered antagonistic: the two inhibitors perform worse together than either inhibitor on its own. An ΣFIC_{min} value ≤0.50 is considered synergistic: the

inhibitors perform better together than the sum of the individual inhibitors on their own (Garcia, 2010b). When the ΣFIC_{min} is ≥0.50 and <4.0, the combination of inhibitors is considered indifferent or additive and there is neither an antagonistic nor a synergistic effect (Garcia, 2010b). Based on these guidelines, there are two combinations that can be considered synergistic: the combination of nisin and Isostab® against *La. plantarum* G326 (ΣFIC_{min} = 0.38), and the combination of nisin and Lactrol against *Lc. lactis* (ΣFIC_{min} = 0.50). None of antimicrobial combinations studied had antagonistic properties; the majority of the combinations exhibited additive or indifferent interactions.

Table 4-4 Sum of fractional inhibitor concentration for combinations of inhibitors in checkerboard experiments against beer spoilage organisms

Strain	Sum of Fractional Inhibitory Contributions					
	Nisin + Isostab®		Nisin + Lactrol		Isostab® + Lactrol	
	ΣFIC_{min}	ΣFIC_{max}	ΣFIC_{min}	ΣFIC_{max}	ΣFIC_{min}	ΣFIC_{max}
Lactobacillus brevis N104	1.00	2.00	1.00	2.00	0.75	2.00
Lactobacillus brevis G433	0.54	1.25	1.00	2.00	1.00	2.00
Lactobacillus plantarum G326	0.38	2.00	0.63	1.25	0.54	2.00
Lactobacillus buchneri N214	1.00	2.00	0.75	2.00	0.54	1.25
Lactococcus lactis MG1363	0.56	1.50	0.50	1.25	0.75	2.0

In combination with leucocin A, nisin gave a fractional FIC_{min} value of 0.50 for three strains: *Lactobacillus brevis* N104, *Lactobacillus plantarum* G326, and *Lactococcus lactis* MG1363 (Table 4-5). This indicates that there is synergy between nisin and leucocin A in some strains because leucocin A has no effect on liquid cultures when used alone. Isostab® gave a FIC_{min} value of 0.50 for *Lactobacillus brevis* G433 in combination with leucocin A; this also may indicate that there is a synergistic interaction.

Table 4-5 Fractional inhibitor contributions for inhibitors in combination with 160 AU/mL leucocin A in checkerboard experiments

Strain	Fractional Contribution of Inhibitor in Combination with Leucocin A					
	Nisin		Isostab®		Lactrol	
	FIC_{min}	FIC_{max}	FIC_{min}	FIC_{max}	FIC_{min}	FIC_{max}
Lactobacillus brevis N104	0.50	1.0	1.0	1.0	1.0	1.0
Lactobacillus brevis G433	1.0	1.0	0.50	1.0	1.0	1.0
Lactobacillus plantarum G326	0.50	1.0	1.0	1.0	1.0	1.0
Lactobacillus buchneri N214	1.0	1.0	1.0	1.0	1.0	1.0
Lactococcus lactis MG1363	0.50	1.0	1.0	1.0	1.0	1.0

4.3.3 Growth curve and shake flask scale up

The combination of nisin and Isostab® was selected for further study because it gave the lowest ΣFIC_{min} value of 0.38, where the individual FIC contributions were 0.25 and 0.13, respectively. Nisin and Isostab® were added individually or in combination at various concentrations to shake flasks containing *La. plantarum* G326. *La. plantarum* counts were recorded at 0, 6, 24, 48, and 72 h (Figure 4-1). The growth of cultures containing 0.5X and 1.0X Isostab® alone displayed an initial growth rate (0-6 h) that was slower than the control, which was grown without inhibitors. The Isostab® containing cultures also reached a lower total cell count than the control and the cultures that contained nisin alone. Nisin had a bacteriocidal effect, characterized by a decrease in cell count for all cultures that contained nisin during the first 6 h of growth, followed by a lag in growth until the 24 h time point. The cultures that

contained 0.5X and 1.0X nisin reached the same final cell density as the control culture, while the culture containing 0.5X nisin and 0.5X Isostab® had a lower final cell density.

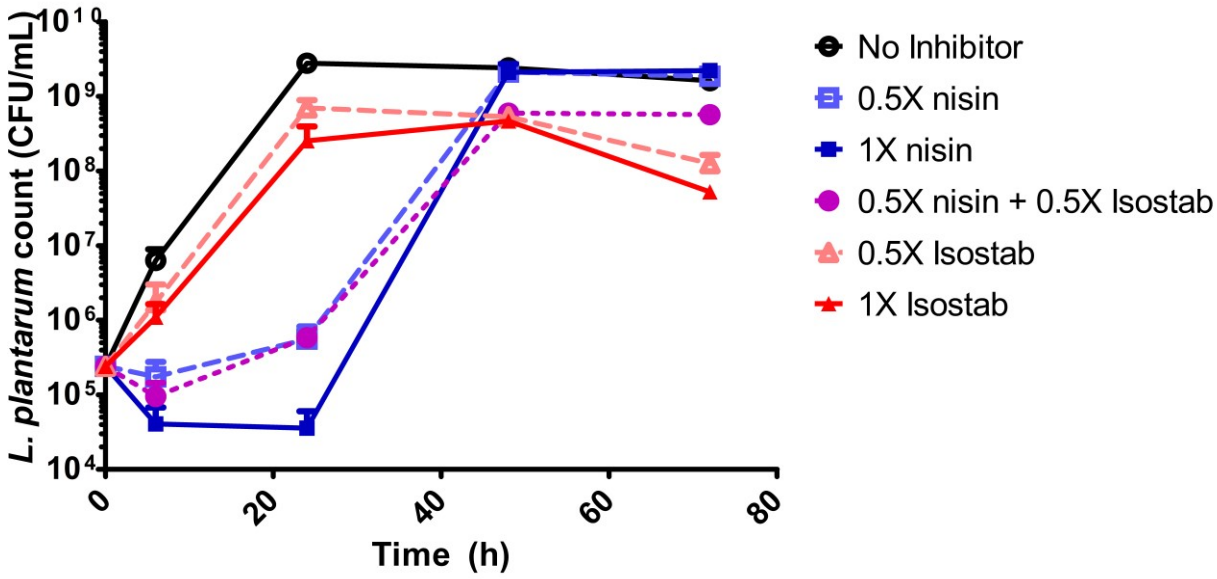


Figure 4-1 Cell counts of *La. plantarum* G326 grown in shake flasks (APT media) fitted with gas traps, in the presence of nisin and/or Istostab®

4.4 Discussion

Hop compounds act as ionophores in the cell membrane and dissipate the proton motive force (Simpson, 1993). We found that both of the *Lactobacillus brevis* strains used in this study exhibited better resistance to the hop-derived Isostab® compared with values previously reported for other *La. brevis* strains (Ruckle & Senn, 2006). This was anticipated since our *La. brevis* isolates were previously found to be relatively hop-resistant (Matoulkova, Sigler, & Nemec, 2010). The *La. buchneri* and *La. plantarum* strains used in this study were also part of the hop resistance study by Maltoulkova *et al.* (2010) and were found to be less hop resistant than the *La. brevis* strains. This is consistent with our findings.

In addition to hop-resistance, all four *Lactobacillus* strains used in this study exhibited more resistance to nisin compared to levels reported in the literature for *Lactobacillus spp,* although these studies cannot be directly compared because the experimental designs were not identical (M. A. Franchi et al., 2012; M. Franchi et al., 2003; Turgis et al., 2012). Nisin works by permeabilizing the cell membrane and causing cell leakage, thereby disrupting the proton motive force (Driessen et al., 1995). Since nisin and hop compounds both function by disrupting the cell membrane, it is likely that the mechanisms of resistance to these compounds share common features. It has been reported that a shared mechanism of resistance for nisin and hop compounds is the modification of the cell wall (Behr et al., 2006; MaisnierPatin & Richard, 1996). In addition, cross-resistance between hop compounds and nisin in the strain *Lactobacillus brevis* ABBC45^{CR} has been attributed to the presence of a multi-drug resistance pump (Suzuki, Sami, Kadokura, Nakajima, & Kitamoto, 2002). It is possible that similar mechanisms could be involved in the relative hop and nisin resistance of the *Lactobacillus* strains examined here.

While a multi-drug resistance pump may be present in the *Lactobacillus* strains used in this study, the relative sensitivity to Lactrol® indicates that other mechanisms of cross-resistance between nisin and hop compounds may be at work. If a multi-drug resistance pump were present, the strains may be expected to be much less sensitive to the drug, although multi-drug resistance pumps have varying degrees of specificity towards antibiotics. For example, the multi-drug resistance pump that is known to confer hop-resistance, HorA (Sakamoto, Margolles, van Veen, & Konings, 2001) is not known to confer resistance to streptogramins like virginiamycin.

The information obtained in this study is not sufficient to explain the molecular mechanisms involved in the resistance, and these explanations are only speculation based on the information available about these strains. In addition, it may be possible that the relative resistance to the hop compounds, nisin, and Lactrol® is unrelated and the observed resistances are due to differences in culture and assay conditions between the studies. A major deviation between the MIC measurement protocols was the use of a spectrophotometer to judge the MIC of the inhibitors, rather than visual inspection that is typically used in studies of this type. Visual inspection was not used in this study because preliminary results indicated that it was difficult to achieve repeatable results with acceptable error.

The *Lactococcus lactis* strain used in this study also displayed relative resistance to both Isostab® and Lactrol®. *Lactococcus lactis* MG1363 contains the gene for an ATP-binding multidrug resistance pump, LmrA (VanVeen et al., 1996). LmrA has not been reported to confer resistance to streptogramin antibiotics nor hop compounds, although it does possess 53% sequence similarity to the HorA hop resistance pump (Sakamoto et al., 2001). Nevertheless, because it is not known to confer resistance to streptogramin antibiotics, it is likely not the source of the strain's resistance properties.

Lactococcus lactis MG1363 was much more sensitive to nisin than the *Lactobacillus spp* studied; however, the former strain was ten times less sensitive to nisin than reported literature values for this strain (Kramer et al., 2006). This is consistent with the findings above that indicated that the strain was relatively resistant to other inhibitors compared with previous data. The explanation for this resistance is likely that the methodology used to produce the literature value was different from that used in this study, so a direct comparison is not applicable.

Despite being active in a spot on lawn assay for three of the five strains studied, leucocin A gave no inhibition against any of the strains in the MIC turbidity measurement experiment. This is contradictory to previous literature where turbidity measurements for bacteriocin activity in liquid culture were more sensitive to low levels of bacteriocin than spot on lawn assays (Papagianni, Avramidis, Filioussis, Dasiou, & Ambrosiadis, 2006; Parente, Brienza, Moles, & Riccardi, 1995; Turcotte, Lacroix, Kheadr, Grignon, & Fliss, 2004). For example, in the most recent of these studies where the lower detection limits of the spot-on-lawn assay were compared with a turbidity measurement, Papagianni *et al* (2006) found that in *Lactobacillus* spp., the minimal detectable amount of nisin is approximately 10 IU/mL (concentration of the liquid used to create the spot) in a spot-on-lawn assay, compared with 1 IU/mL in a turbidity measurement assay. Because these findings that liquid turbidity assays were more sensitive to low levels of bacteriocin than spot-on-lawn assays have been consistently reported for the Class I and Class IIa bacteriocins assayed, but not leucocin A, there may be some property unique to leucocin A that makes it less active in liquid media than on an agar plate measured in the spot on lawn assay in this study. The low activity of leucocin A in liquid cultures may also play a role in the low apparent leucocin A activity in Chapter 2.

Antimicrobial synergy requires that antimicrobials have mechanisms of action that are unique from one another, yet the mechanisms of action are related in a way that allows their combined effect to be greater than the sum of each inhibitor on its own (Denyer et al., 1985). If antimicrobials act through a similar mechanism, but the mechanisms do not complement each other, the relationship is considered additive or indifferent (Denyer et al., 1985). Nisin and Isostab® was the only combination of inhibitors in this experiment to give a ΣFIC_{min} value <0.50 in a checkerboard experiment, indicating that they may be working synergistically. However, the ΣFIC_{max} value was 2.00, which is higher than the other strains. This range reflects the variability experienced between replicates of the experiment and may suggest that the measured synergy may be due to the variability. Performing a shake flask experiment was critical to confirm whether this combination was synergistic, or an additive combination that produced variable experimental results.

The shake flask time course study on *La. plantarum* indicated that the relationship between nisin and Isostab® is additive, rather than synergistic, and did not work better than nisin alone. This lack of synergy is especially apparent at the 24 h time point, which is the time point closest to the 20 h time point used in the checkerboard experiment. There are a number of reasons why the relationship is additive rather than synergistic in shake flasks, although it appears to be synergistic in the checkerboard assay. As discussed above, the variability in the checkerboard experiment may have played a large role in indicating synergy for the combination of inhibitors while the combination may truly be additive. This combination could be additive because the mechanisms of action are too similar to complement each other in a synergistic fashion. As mentioned above, Nisin and Isostab® both work by dissipating the pH and ionic gradient of the cell.

A second reason that the assay may show synergy in the checkerboard assay but not in the shake flask experiment is that the two experiments are not directly comparable. Although the conditions are similar, the mixing, oxygen concentration (checkboard assay was done in a sealed 96 well plate, while shake flasks were capped with a gas trap, so different oxygen concentrations may have resulted from these different setups), and shear stress on the cells would all be different in the shake flask and the MIC determined for each experiment is likely different. This reinforces the need for scale up of assays that are going to be used in industry prior to their

application, because results that are promising a small scale may not be as relevant at a large scale.

A final reason that the assay may appear to be less synergistic in the shake flask, especially over a longer time course, is that the cells may be breaking down the inhibitors, or the inhibitors may be breaking down in the media, allowing the cell population to recover. This could be studied further. If there is evidence that breakdown of the inhibitors is occurring, it may be best practice for industry to add a continuous dose or frequent doses of the inhibitors in order to control the bacterial population.

The results of the modified checkerboard experiment using a combination of leucocin A and nisin indicate that this combination is synergistic; leucocin A gave no inhibition on its own, but improved the activity of nisin for three strains in several replicates. Synergy between nisin and pediocin PA-1, a class IIA bacteriocin, has been previously reported (Bennik et al., 1997; Hanlin, Kalchayanand, Ray, & Ray, 1993). The synergy between these bacteriocins could be applied to an industrial spoilage system if class IIA bacteriocins could be produced at an industrially-relevant cost, either by creating a strain of fermentation yeast that efficiently secretes class IIA bacteriocins, or by purifying leucocin A from a culture of the producer organism. However, it is important to note that *Listeria monocytogenes* strains resistant to nisin can have cross-resistance to class IIa bacteriocins (Naghmouchi, Kheadr, Lacroix, & Fliss, 2007), and that this cross-resistance phenomena could extend to fermentation spoilage strains. The mechanism of this cross-resistance has not been reported.

The modified checkerboard experiment also suggests that the combination of leucocin A and Isostab® may exhibit synergy. The combination of leucocin A and Isostab® against *La. brevis* G433 also gave a ΣFIC_{min} value of 0.50. This relationship should be studied further in a shake flask assay.

In order to generate cost savings in an industrial context, the inhibitor combinations should be synergistic rather than additive or indifferent, so that less total inhibitor can be added. An additive effect may generate marginal cost savings if there is a significant difference between the cost of the two inhibitors, allowing for the lower cost inhibitor to replace the activity of a portion of the higher cost inhibitor in the fermentation. The combination of nisin and Isostab® is

promising, but it needs to be further optimized so that the hurdle technology (Leistner & Gorris, 1995) could be used to better control contamination events. Contamination events during ethanol fermentation typically involve multiple strains of lactic acid bacteria (Lucena et al., 2010). As reinforced by this study, these strains will likely have varying degrees of sensitivity to microbial inhibitors. For example, *Lactococcus lactis* MG1363 is highly sensitive to nisin relative to the other strains studied, but was also highly resistant to Lactrol®. Using Lactrol® alone at the manufacturer's suggested dosage may be effective against the *Lactobacillus* strains studied, but may not be effective against this *Lactococcus lactis* strain. Using a combination of nisin and Lactrol would control both the Lactrol-sensitive and the Lactrol-resistant strains used in this study because the Lactrol-resistant strain exhibits a unique sensitivity to nisin.

Combinations of three or more inhibitors were not studied, but may also be useful in controlling contamination, particularly if resistant bacteria are present. Although this would increase the complexity of the study, it may have a tremendous benefit to industry. In particular, there may be a significant benefit to ethanol plants where multiple inhibitors are already used because inhibition could be optimized for cost-savings.

Chapter 5: A set of vectors for ligation-independent cloning in *Saccharomyces cerevisiae*

5.1 Introduction

DNA manipulation techniques pioneered in the 1970s and 1980s, specifically the use of the polymerase chain reaction (PCR) to amplify genes of interest (Saiki et al., 1985) and the restriction digest and ligation methodology used to assemble plasmids (Cohen, Chang, Boyer, & Helling, 1973) are still prevalent techniques in molecular biology today. In yeast, these techniques are used to clone genes into 5 categories of vectors, classified by the way that the vector is replicated and maintained within the cell; each type of vector has desirable and undesirable properties depending on the experiment being performed (Lundblad, 2001). Two of these vector categories: yeast centromere plasmids (YCps) and yeast integrating plasmids (YIps) are used in this study.

YCps contain an autonomously replicating sequence to drive replication of the vector and a centromere sequence that allows for partitioning of the replicated plasmids between daughter cells during cell division (Gietz & Sugino, 1988). YCps typically have low copy number and low loss rate (Gietz & Sugino, 1988). YC plasmids are initially selected for using an auxotrophic marker (Gietz & Sugino, 1988).

YIps are plasmids that, after the sequence of interest is cloned into the plasmid, integrate into the genome of the yeast cells through homologous recombination (Sikorski & Hieter, 1989a). Homologous recombination typically occurs at a site within an auxotrophic marker, which allows for selection of successful clones (Sikorski & Hieter, 1989). This results in a single copy of the gene that is very stably maintained (Sikorski & Hieter, 1989).

Assembly of both YC and YI plasmids traditionally occurred using the restriction digest-ligation method to assemble the plasmid, the plasmid sequence was confirmed in *E. coli*, and then the vector was shuttled to *S. cerevisiae* (Cohen et al., 1973). This method has two major disadvantages. Because the methodology relies on restriction digests to create compatible ends on the insert sequence and the vector, the insert must be free of the restriction enzymes sites used in the cloning process. Another drawback of this method is the ligation step, which is notoriously unreliable (Speltz & Regan, 2013).

Several alternative methodologies have been developed to replace restriction digest-ligation cloning. In yeast, these methodologies typically use the cell's homologous recombination system to generate the final cloning product. Yeast recombinational cloning - where insert DNA is flanked by 20-200 bp regions with homology to a cloning site in the vector DNA is co-transformed into competent cells with vector DNA, resulting in the insert being inserted into the cloning site by the cell's homologous recombination machinery - was first used by Ma *et al* (1987) and optimized by Oldenburg *et al* (1997). The protocol developed by Oldenburg *et al* (1997) used a single insert containing a 40 nucleotide overlapping sequence with the insertion point and found that 69% of transformations were successful. This cloning strategy was adopted by many labs and was famously used in the final step to assemble the final four quarter chromosome sections for the first complete synthetic assembly of the *Mycoplasma genitalium* genome (Gibson et al., 2008). When assembling a large number of DNA parts, this method gives a range of 10-80% positive clones (Kuijpers et al., 2013).

Gibson (2009) developed a cloning methodology that uses homologous recombination machinery to join regions of homology between many adjacent single stranded DNA oligonucleotides and a double stranded plasmid following transformation of the single stranded DNA and the double stranded plasmid. Joska *et al.* (2014) recently modified this methodology to allow for the yeast homologous recombination system to be used to generate vectors for any organism by co-transforming and recombining yeast centromeric and selection markers into the organism-specific plasmid while transforming and recombining the sequence of interest. This allows for the *S. cerevisiae* recombination machinery to be used; then the vector is purified and used in the organism of choice (Joska et al., 2014).

In 2009, Gibson claimed that up to 38 overlapping single stranded molecules could be assembled into a vector using his methodology (Gibson, 2009). Recent studies have shown that this assembly method does not assemble more than four DNA parts with 50% of clones being correct (Kok et al., 2014). This makes the Gibson method somewhat inefficient and impractical for large constructs built from many small DNA fragments.

Two alternative methodologies have been developed to improve the positive transformation rate for the assembly of constructs with a large number of DNA parts. The ligase cycling reaction uses short oligonucleotide bridges annealed to two adjacent DNA parts in order

to hold the parts adjacent to each other, and ligase is added to join the DNA parts; this reaction sequence of annealing and ligase addition is repeated for many cycles (Kok et al., 2014). The ligase cycling reaction was used to join up to 12 DNA parts with 60-100% positive clones generated (Kok et al., 2014). The second alternative methodology that has been recently been used to generate 95% positive clones from 9 DNA fragments (Kuijpers et al., 2013). This strategy uses 60 bp synthetic homology regions rather than relying on yeast homology regions or regions within the DNA parts, this allows for very specific recombination (Kuijpers et al., 2013). It also separates the selection and centromeric sequences in the vector, to prevent false positive clones generated by backbone cyclization (Kuijpers et al., 2013).

While these developments are quite promising, they are also somewhat limited by the number of DNA fragments present. Although the reason that is a low percentage of desired constructs created when high numbers of DNA fragments are combined has not been studied, one can hypothesize that one limitation is the requirement that all of the DNA fragments enter the cell, which could be problematic with larger numbers of fragments. A methodology where single stranded, complementary overhangs on adjacent DNA fragments are created and DNA is annealed prior to transformation may improve the likelihood of large numbers of fragments successfully transforming into the cell because the hydrogen bonding between the strands may ensure that the DNA strands are co-transformed into the cell.

All of the alternatives to restriction digest-ligation cloning discussed above can be considered ligation-independent cloning (LIC); however, in this study LIC will refer to the procedure discussed below. LIC was first used to successfully clone PCR products into *Escherichia coli* expression vectors (Aslanidis & de Jong, 1990). This methodology is presented in Figure 5-1. The advantage of this methodology in the context of *E. coli* expression vectors is that in LIC, PCR products of the desired gene sequence are designed with specific sites on the both ends of the molecule that generate overhangs that are complementary to overhangs on the vector. When the PCR product is incubated with a 3' exonuclease and a specific dNTP, the reaction generates 12-20 bp that single-stranded overhangs that pair with the vector overhangs. The vector is designed to contain complementary ends after it is linearized at the cloning site and treated with a 3'exonuclease and the dNTP complementary to the one used in the PCR product

treatments. The treated insert and vector are then mixed at room temperature, the complementary overhangs anneal, and the mixture is transformed into *E. coli*.

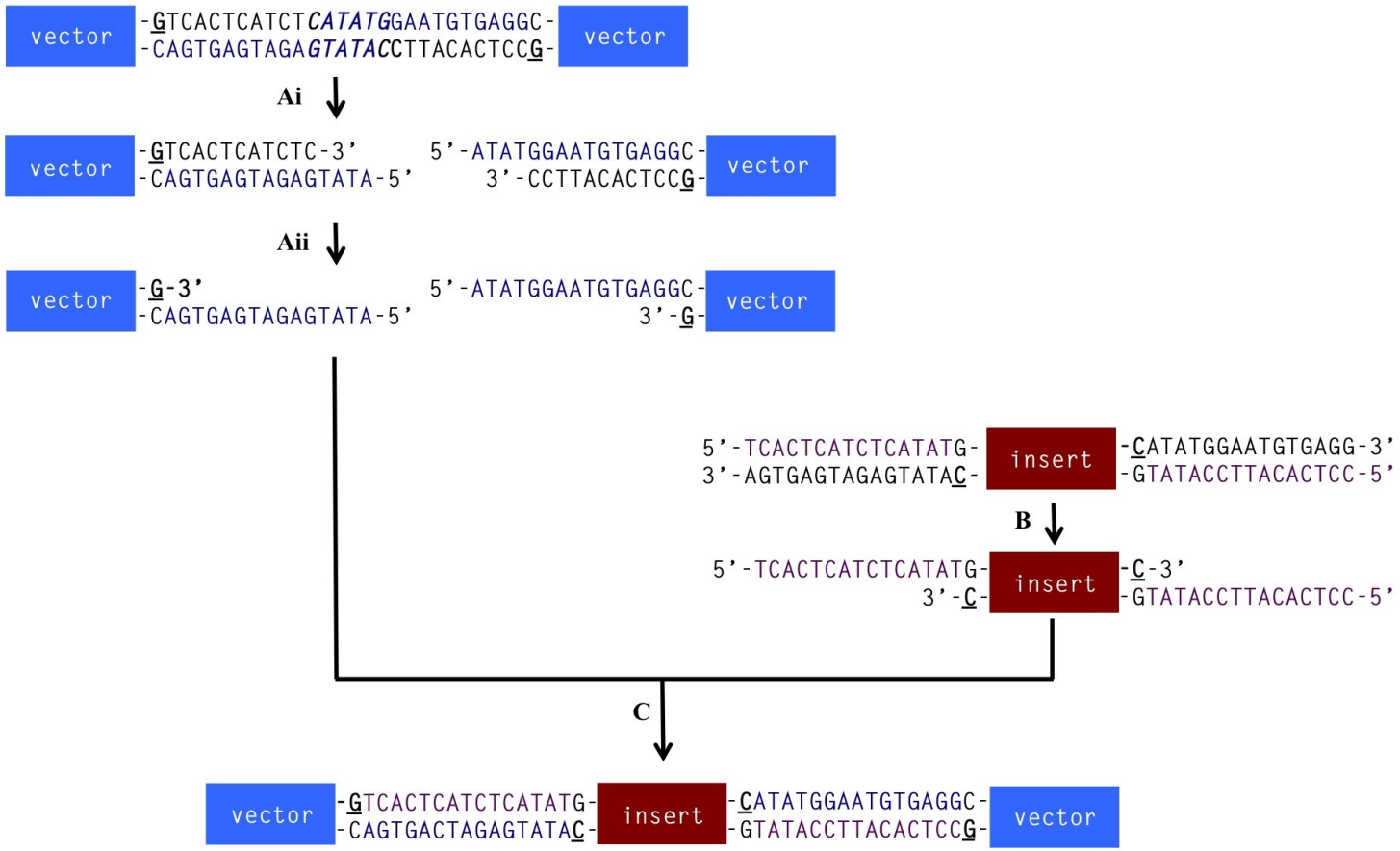


Figure 5-1. LIC system used in this chapter. In Step Ai, the vector is treated with the restriction enzyme *Nde*1 (italicized), which cuts at the CATATG site in the molecule. The vector is then treated with dGTP and a 3' exonuclease, which generates 15 bp single stranded tails on the molecule (shown in blue). In Step B, the insert PCR product is treated with dCTP and a 3' exonuclease, which generates 15 bp single stranded tails on the molecule (shown in purple) that are complementary to the single stranded tails on the vector. In step C, vector and insert are combined and the complementary single stranded tails anneal.

This style of LIC has not been performed directly in yeast, possibly because the methods discussed above are often used and are ligase-independent; it has only been used to generate yeast vectors first by LIC and transformation into *E. coli* via a yeast-*E. coli* shuttle vector, followed by purification of the vector and transformation into *S. cerevisiae* (K. M. Clark et al., 2010). One disadvantage of using LIC to create plasmids in *E. coli* and then transforming the constructs into *S. cerevisiae* is that the shuttling step between *E. coli* and yeast takes extra time to perform. Using this system to perform LIC directly in yeast has the added advantage of bypassing the vector shuttling between *E. coli* and yeast and therefore saving time in the cloning process.

There are two major advantages to this cloning methodology over the conventional restriction digestion/ligation method:

- The process is agnostic to restriction enzymes present in the insert of interest. This means restriction enzyme sites do not need to be removed from the insert DNA prior to cloning.
- The process does not depend on the ligation step, which has been described as unreliable (Speltz & Regan, 2013).

In addition to the advantages over the restriction digest-ligation cloning, LIC techniques may improve cloning by homologous recombination by improving transport of all DNA parts into the cell, which is particularly relevant when a large number of DNA molecules are being cloned simultaneously.

The goal of this study is to generate LIC vectors for direct cloning into *S. cerevisiae*.

5.2 Materials and Methods

5.2.1 Culture Maintenance

Bacterial strains (Table 5-1) were grown in Luria-Bertani (LB) Broth with appropriate antibiotics (Difco™; Becton Dickenson; Sparks, MD). Yeast strains were grown for selection in Complete Minimal (CM) Media (Ausubel, 1992) without the appropriate amino acid for

selection [Yeast Nitrogen Base-AAS (Sigma-Aldrich; St. Louis, MO, USA), ammonium sulfate (Thermo Fisher Scientific; Waltham, MA, USA), dextrose (Thermo Fisher Scientific), amino acids (Sigma-Aldrich)], and Yeast Extract Peptone (YP) media (Ausubel, 1992) [(BD Yeast Extract (Becton Dickinson), BD Peptone (Thermo Fisher Scientific); (Ausubel, 1992) with raffinose (YP-Raff; Sigma-Aldrich), dextrose (YP-Glu; Thermo Fisher Scientific), or galactose (YP-Gal; Sigma-Aldrich)] for growth, non-induction, and induction of P_{GAL1} transcription, respectively. All strains were stored at -80°C in 20% (v/v) glycerol (Sigma-Aldrich).

Table 5-1 Organisms used in this study and their relevant characteristics

Organism	**Characteristics**	**Source or reference**
Saccharomyces cerevisiae W303-1A	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ybp1-1*	(B. Thomas & Rothstein, 1989)
Escherichia coli DH5α™	Molecular cloning	Life Technologies (Grand Island, NY, USA)

Table 5-2 Plasmids and/or DNA used in this study and their relevant characteristics

Plasmid/DNA sequence	**Characteristics**	**Source or reference**
YCplac111 P_{GAL1}	Yeast-*E. coli* shuttle vector, *ARS, CEN4, LEU2*	(Gietz & Sugino, 1988)
YCplac111-P_{GAL1}-LIC-T_{ADH1}	Empty LIC expression vector	This study
YIplac128	Yeast-*E. coli* shuttle vector	(Sikorski & Hieter, 1989)
YIplac211	Yeast-*E. coli* shuttle vector	(Sikorski & Hieter, 1989)
YIplac128-P_{GAL1}-LIC-T_{ADH1}	Yeast integration-LIC vector	This study
YIplac211-P_{GAL1}-LIC-T_{ADH1}	Yeast integration-LIC vector	This study
MFA1-GFP-Leu	GFP source DNA	BioBasic Inc. (Markham, ON, Canada)
pET-11a	Used as template DNA in this study, source of 0.5, 1.0, and 2.5 kb inserts	Novagen (EMD Millipore, Darmstadt, DE)

5.2.2 Subcloning of the ligation-independent cloning site

A forward primer containing the *Bam*HI (Life Technologies, Grand Island, NY) restriction site and the LIC site (abbreviated as LIC_S) shown in Figure 5-1 and a reverse primer containing the *Eco*RI (Life Technologies, Grand Island, NY) restriction site were used to amplify the *S. cerevisiae* alcohol dehydrogenase transcriptional terminator sequence from yeast total DNA (T_{ADH1}). Primer sequences are provided in Table 5-3. The LIC-T_{ADH1} PCR product was then cloned into the *Bam*HI and *Eco*RI sites of YCplac11 P_{GAL1} using the Thermo Scientific Rapid Ligation Kit (Thermo Scientific, Pittsburgh, PA, USA). Positive clones were confirmed by restriction digest and gel electrophoresis, followed by sequence confirmation using Sanger sequencing using the appropriate primers and the BigDye® Terminator Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and sequenced on the ABI 3730 Sequencer (Life Technologies). The resulting plasmid was named YCplac111-P_{GAL1}-LIC-T_{ADH1}.

P_{GAL1}-LIC-T_{ADH1} was amplified from YCplac111-P_{GAL1}-LIC-T_{ADH1} using the primers indicated in Table 5-3 and cloned into the *Bam*HI and *Eco*RI sites of YIplac128 and YIplac211 using the same methodology as the LIC-T_{ADH1} cloning described above.

Table 5-3 PCR primers used in this study (Chapter 5). Restriction sites for subcloning using restriction digest-ligation indicated in orange italics. LIC homology regions indicated in blue for vector and red for insert, with the *Nde*1 site used for LIC cloning of the vector indicated in italics.

Gene product	Template		Primer sequence (5' to 3')
LIC T_{ADH1}	*S. cerevisiae* total DNA	N	CAT*GGATCC*GTCACTCATCT*CATATG*GAATGTGAG GCGCGAATTTCTTTATG
		C	CAGTCAAGGTATGATTCGTACG*GAATTC*GGGAGCG ATTTG
P_{GAL1}-LIC-T_{ADH1}	YCplac111	N	CAC*AAGCTT*GCATGCCCCATTATCTTAGC
	P_{GAL1}-LIC-T_{ADH1}	C	CAC*GGATCC*CGGGAGCGATTT
GFP	MFA1-GFP-Leu (See chapter 2)	N	TCACTGATCTCATATCATGTCTAAGGGTGAAGAA
		C	GCCTCACATTCCATATGTTACTTATACAATTCGTC CATA
500 bp	pET-11a	N	TCACTGATCTCATATGTTCTCATGTTTGACAGC
		C	GCCCTCACATTCCATATGTGATGCCGGC
1 kb	pET-11a	N	TCACTGATCTCATATGTTCTCATGTTTGACAGC
		C	GCCTCACATTCCATATGGTTGTTGTGCCAC
2.5 kb	pET-11a	N	TCACTGATCTCATATGTTCTCATGTTTGACAGC
		C	GCCTCACATTCCATATGGTTCCATGTGCT

5.2.3 Ligation-independent cloning into YC vectors

Ligation independent cloning inserts were amplified from template DNA (described in Appendix A) using a forward primer containing the 5' LIC site shown in Figure 5-1 and a reverse primer containing the 3' LIC site shown in Figure 5-1. Inserts were purified using the PCR Purification Kit (Qiagen, Venlo, Limburg, Netherlands). The DNA concentration of the samples was determined using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). 240 fmol of the purified inserts were treated with LIC-qualified T4 DNA polymerase (Novagen, EMD Millipore, Billerica, MA, USA) in 20 µL total volume containing 5 mM DTT (Sigma-Aldrich, St. Louis, MO, USA), 2.5 mM dCTP (Life Technologies), 1X T4 DNA Polymerase Buffer (Novagen), and 0.2 U T4 DNA polymerase (Novagen) for 40 min at 22°C followed by 15 min at 75°C.

Vectors were digested with *Nde*I (Life Technologies, Grand Island, NY) and purified using the PCR Purification Kit (Qiagen). Further processing was achieved using the same methods as described for the LIC inserts with the following changes: 37 fmol of the purified vector was used in the 20 µL reaction instead of 240 fmol, and dGTP (Life Technologies) was used in place of dCTP.

The treated vector and insert were then combined (2.5 µL of each) at room temperature for 5 min, and then 2.5 µL of 25 mM EDTA (Sigma-Aldrich) was added. The mixture was incubated for 5 min at room temperature and then transformed into *S. cerevisiae* W303 cells using a DMSO assisted yeast transformation protocol (Hill et al., 1991). Transformed cells were plated on CM media lacking the appropriate amino acid for selection.

5.2.4 Ligation-independent cloning into YI vectors

The YI vector was prepared for LIC as described for YC vector in 5.2.3. The only deviation is that following treatment to prepare the 5' overhangs, the vector was digested with *Eco*RV to prepare it for homologous recombination. The LIC-ready YI vector (4.6 fmol) and insert (30 fmol, prepared as per section 5.2.3) were combined, transformed, and selected as described in section 5.2.3. For LIC cloning in the YI vector, other ratios of vector:insert were also examined: 46 fmol:300 fmol, and 460 fmol:3000 fmol. In some cases, phenol/chloroform extraction of the mixtures after incubation at room temperature was performed, followed by

precipitation with ethanol, resuspension in 5 µL of MilliQ water, and transformation into *S. cerevisiae* W303 cells using DMSO assisted yeast transformation protocol (Hill et al., 1991)

5.2.5 Analysis of cloning – DNA analysis

Colonies were streaked on selective media and incubated for 48-72 h at 30°C. 5 mL liquid cultures of the appropriate selective media were then inoculated using the streaked colonies and the tubes were shaken at 30°C and 200 rpm for 24-36 h. 1 mL samples were taken for DNA extraction, which were centrifuged (1 500 x *g*, 5 min) to pellet cells. The supernatant was removed and the cells were vortexed (4 x 1 min) in lysis buffer (50 mM Tris, pH 7.5 (Sigma-Aldrich), 20 mM EDTA (Sigma-Aldrich), and 1% SDS (Sigma-Aldrich)) containing 0.5 mm glass beads (Sigma-Aldrich). The mixture was extracted using phenol and chloroform and the DNA was precipitated using ethanol. The precipitated DNA was resuspended in MilliQ water and used as a PCR template with primers targeting the N- and C- termini of the cloned insert. PCR products were separated on a 1% agarose gel.

5.2.6 Analysis of cloning – protein expression

Yeast cultures were grown as described in section 5.2.5. Following centrifugation (1 500 x *g*, 5 min), the cell pellets were resuspended in YP-Gal media to induce expression of the insert protein, green fluorescent protein (GFP). The tubes were shaken at 30°C and 200 rpm for 24 h. The cells were centrifuged (1 500 x *g*, 5 min), washed in 20% trichloroacetic acid (TCA; Thermo Fisher Scientific; Waltham, MA, USA), and then resuspended in 200 µL 20% TCA. 0.5 mm glass beads were added, and the cells were vortexed (4 x 1 min). 400 µL 5% TCA was then added to the tube. The cells were then centrifuged (16 000 x *g*, 10 min) and the resulting protein pellets were washed 3X with acetone (Fisher). The protein pellets were then resuspended in SDS PAGE sample buffer and subjected to SDS PAGE and Western blotting (anti-GFP antibody, GE Life Sciences, Piscataway, NJ).

5.3 Results

5.3.1 Preparation of LIC vectors

The vectors were prepared for LIC by placing a LIC site between an inducible promoter (P_{GAL1}) and transcriptional terminator (T_{ADH1}) (Figure 5-2, plasmid map show in Figure 5-3). The LIC site contains a restriction site (*Nde*I) and an absence of cytosine in the sequence for 12 bases upstream and an absence of guanine residues downstream of the site (Figure 5-1). This allows for the generation of 15 nucleotide 5' overhangs on both ends of the vector when incubated with a 3' exonuclease and dGTP. The exonuclease activity stops at the first 3' guanine residue because dGTP is available in the reaction mixture and thus the removal and replacement of guanine residues are in equilibrium.

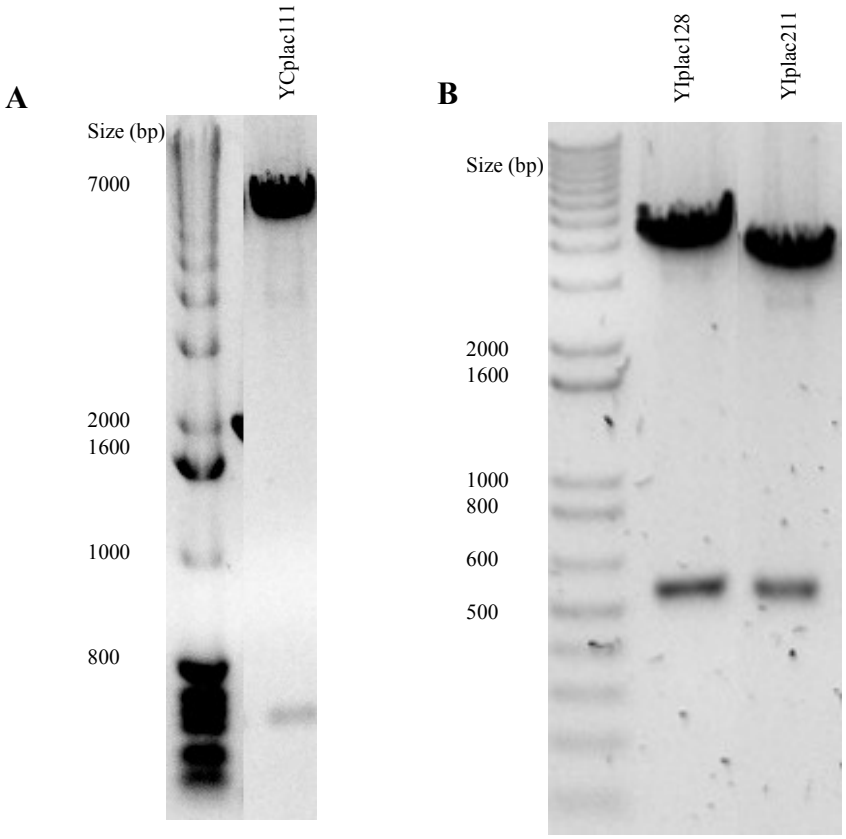


Figure 5-2 Cloning products for creation of the LIC-ready protein expression vectors: *CEN* (YC) vector (A) and integrating (YI) vectors (B)

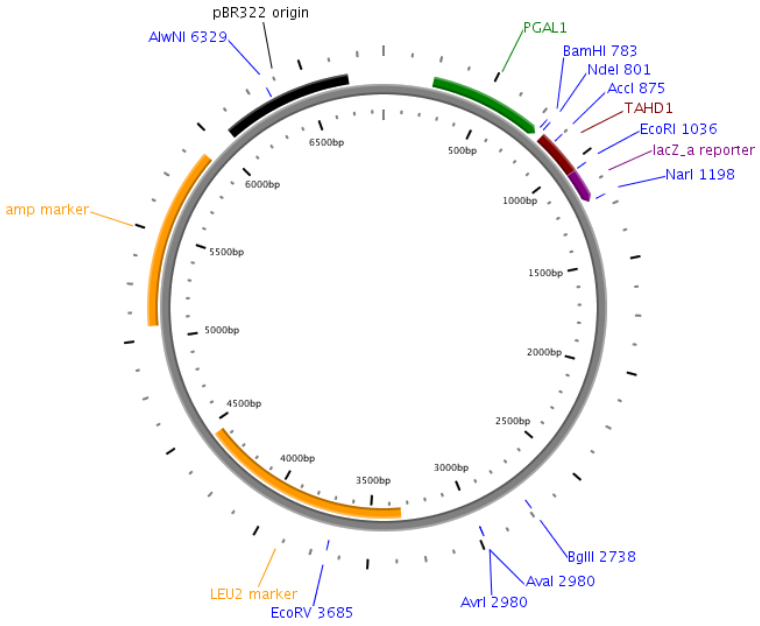


Figure 5-3 Plasmid map of YCplac111-P$_{GAL1}$-LIC-T$_{ADH1}$ generated using PlasMapper (Dong et al., 2004)

5.3.2 LIC with YC vectors

The insert, green fluorescent protein (GFP), was amplified using primers that introduced flanking LIC sites. The flanking LIC sites on the insert are designed to create complementary 15 bp overhangs with the vector when the insert is incubated with a 3'exonuclease and dCTP. When the treated insert and vector are combined, the complimentary overhangs overlap and generate an unclosed circular plasmid. The plasmid was transformed into *S. cerevisiae,* plated on the appropriate selective media for the plasmid, and colonies selected for screening. The colonies were screened for the presence of the plasmid using PCR for the GFP gene and for galactose-induced GFP expression by Western blot (Figure 5-4). Of the ten colonies screened in this experiment, seven were positive for both insert presence in the DNA and protein expression. This indicates that the system can successfully be used

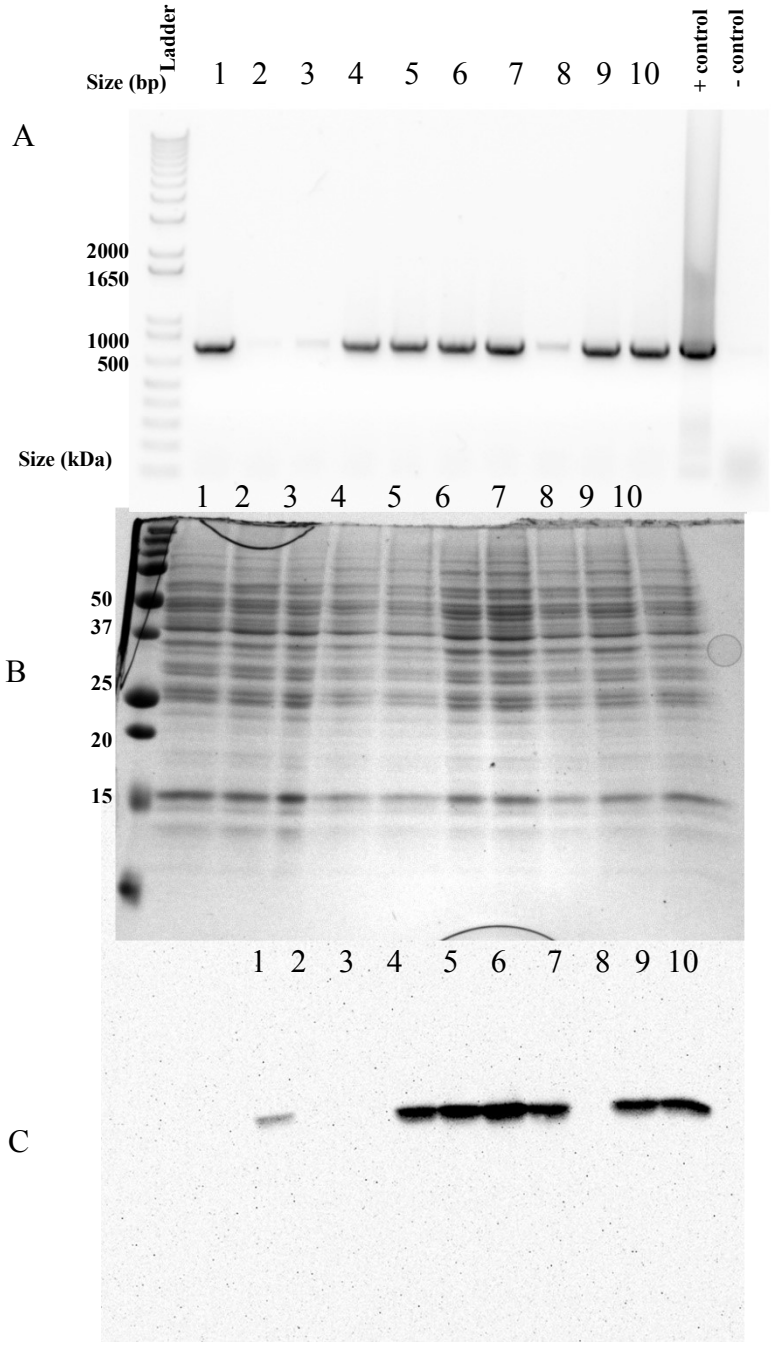


Figure 5-4 Agarose gel (A), SDS-PAGE (B), and Western blot (anti-GFP) (C) results from colony screening after LIC cloning of GFP.

The same LIC system was then used to clone three different sized inserts in order to test if the system could be used for inserts other than GFP. DNA extraction and PCR analysis of colonies indicate that the LIC system can be used successfully with inserts of different sequence and size (Figure 5-5). The results shown in Figure 5-5 are positive results of a single colony. LIC for each insert was repeated in triplicate and twenty colonies were screened per experiment, the results for this experiment are summarized in Table 5-4. The percentage of positive colonies of the total colonies screened for each experiment was highly variable, ranging from 10% to 85%. Means ranged from 23 to 72%, with standard deviations of up to 33% (Table 5-4). There is no apparent effect of the GC content or the size of the insert on the rate of positive clones.

Table 5-4. Mean, standard deviation, and GC content for percentage of positive clones cloned using the LIC methodology, 20 clones were analyzed per experiment, n=3

Insert	**Mean**	**Standard Deviation**	**GC content**
	(% Positive)	**(% Positive)**	**(% GC)**
550 bp	37	33	49
1 kb	23	15	54
2.5 kb	43	25	56
GFP	72	23	39

A suggested protocol for LIC in YC vectors is presented in Appendix D.

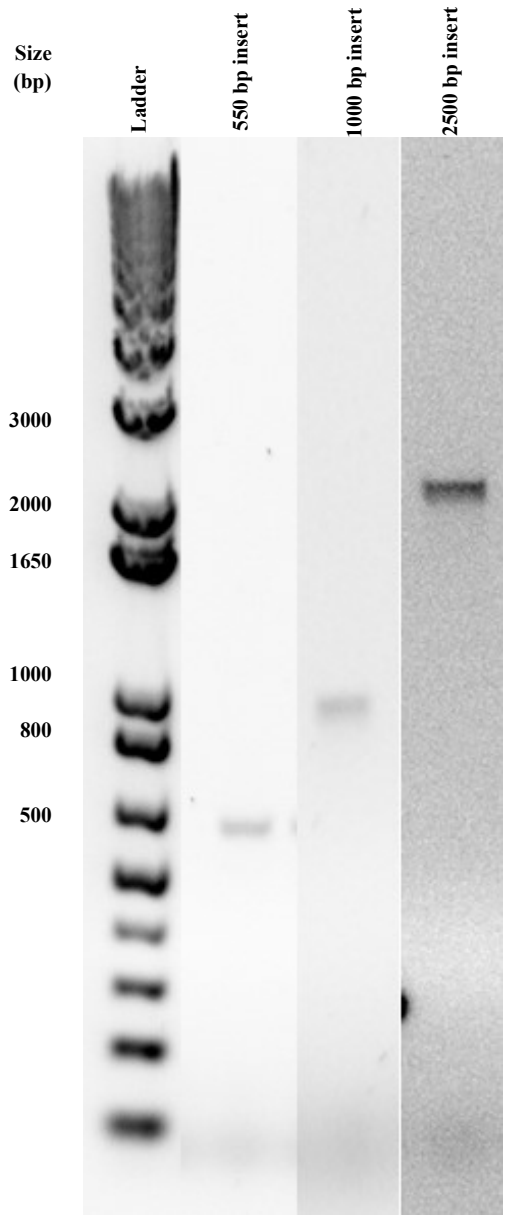


Figure 5-5 Agarose gel of positive clones of three different sized inserts (0.55 kb, 1.0 kb, 2.5 kb) cloned using LIC system.

5.3.3 Attempts at LIC with YI vector

The LIC system was then tested using integrating vectors rather than circular vectors. The vectors were generated by cloning the P_{GAL1}-LIC-T_{ADH1} sequence into the integrating vectors YIplac128 (Figure 5-2, plasmid map shown in Figure 5-6). The vector was prepared for LIC by creating the LIC overhangs, purifying the DNA, and then digesting the DNA with *Eco*RV to create a double stranded break within the auxotrophic marker and purifying to generate a linearized vector (Figure 5-7). For integration vectors, the double stranded break at the *Eco*RV site within the auxotrophic gene serves as a recombination site for the vector within the host DNA.

The vector and insert prior to the LIC reaction are shown in Figure 5-7. The LIC-ready GFP insert was then combined with the LIC-ready and linearized integrating vectors, transformed into *S. cerevisiae* W303 cells, and plated on selective media. The transformations yielded no colonies. The molar concentration of the vector and insert were increased 10 and 100 fold to improve the likelihood of cloning and integration; this increase in concentration did not generate colonies for screening.

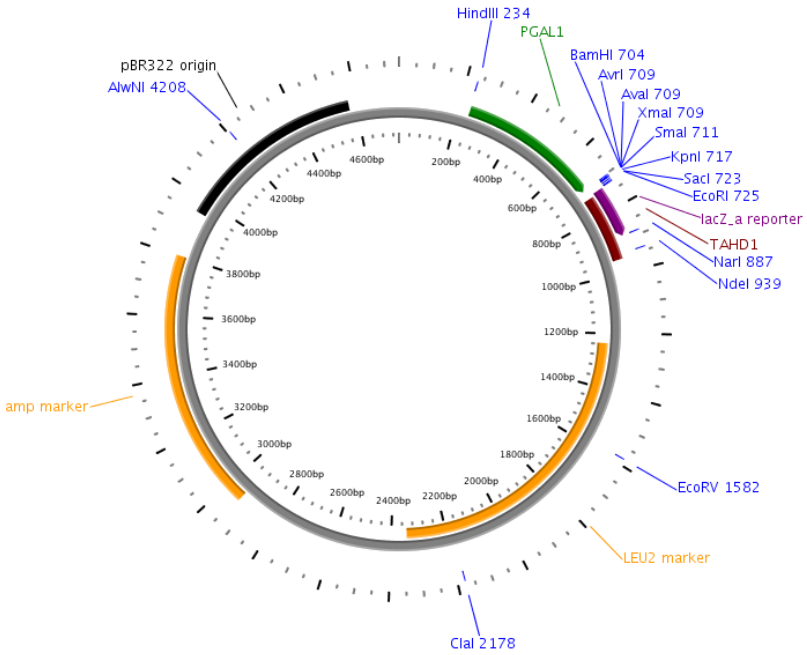
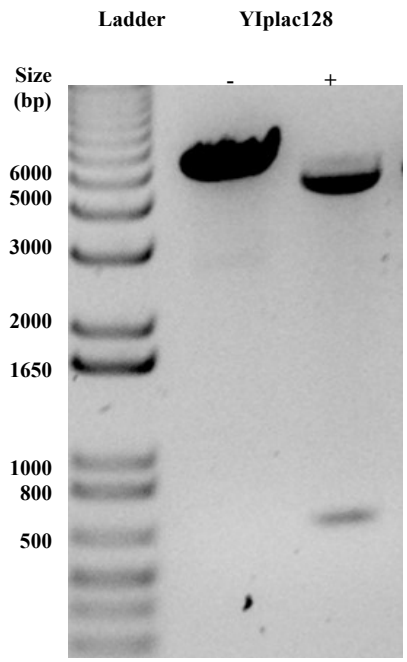


Figure 5-6 Map of YIplac128-P$_{GAL1}$-LIC-T$_{ADH1}$ created in this study. Plasmid map generated using PlasMapper (Dong et al., 2004).



Expected Fragment Size	**4100**
(restriction fragment prediction analysis)	**642**

Figure 5-7 LIC-ready yeast integration vector and inserts prior to LIC and transformation.

5.4 Discussion

The YC vectors developed in this study, along with the protocol presented in Appendix D, can be used for LIC of inserts in *S. cerevisiae*. This direct-to-yeast protocol offers significant time-savings over conventional cloning and LIC through yeast-*E. coli* shuttle vectors. This protocol is advantageous over convention restriction digest-ligation cloning because the procedure is agnostic to the presence of restriction sites within the insert DNA and does not rely on the ligation reaction.

The protocol developed in this thesis generates 10% - 85% positive colonies per experiment; there is large standard deviation of percent positive colonies. There was no correlation between insert size and success rate. There was also no correlation between GC content and success rate. Higher GC content has been shown to increase the rates of recombination within *S. cerevisiae* DNA (Gerton et al., 2000), but this was not the case for this experiment.

One reason why success rates for this process may be inconsistent is that although all reasonable measures were taken to ensure that experimental details steps were followed in a repeatable manner, experimental replicates were performed on different days and transformation-competent cells were created from different yeast cultures. Two particular steps of yeast transformation may have a high degree of variability: a repeatable cell count when cells are harvested for the procedure because the cells are in the logarithmic growth phase, and the mixing of the cells and DNA material within the PEG solution is limited due to the viscosity of the solution. These reasons for poor repeatability may also be the reason for the poor repeatability found by Kok *et al.* (2014) when repeating the Gibson (2009) cloning methodology.

We were hoping that creating overhangs that allowed the molecule to potentially form prior to transformation into yeast would result in higher rates of successful cloning than those established in 2009 (Joska et al., 2014; Kok et al., 2014; Kuijpers et al., 2013). While we showed that our method could be effectively applied for the cloning of yeast plasmids, we could not confirm that our method was any better than cloning by homologous recombination or other cloning methods developed after 2009. However, situations may exist where our LIC style of cloning may be a more advantageous cloning technique.

We were not able to develop a set of YI vectors and a protocol for LIC and simultaneous integration of inserts in *S. cerevisiae*. One potential reason that the cloning was unsuccessful is that the non-covalently linked DNA at the ligation independent cloning site becomes unwound and disassociates during homologous recombination. Another possible reason that the cloning did not work is that the combined frequency of LIC interaction and integration is too low to achieve successful cloning and transformed colonies.

During homologous recombination, the recombination machinery, which includes a 5' to 3' exonuclease, acts on the double stranded break to create long, overhanging single-stranded tails (White & Haber, 1990). These tails can extend up to 1 kb from the double stranded break site; 68% extend past 260 bp, while 35% extend past 601 bp (Sun, Treco, & Szostak, 1991). In the YI vectors designed and used in this study, the LIC site is approximately 1kb from the *Eco*RV cut site. This means that the exonuclease could potentially remove the DNA on one strand up to the nick and the DNA complementary strand could be lost.

The distance from the *Eco*RV cut site to the LIC is approaching the observed limit (1 kb) for the length of the single stranded tails. Even if the single stranded overhang doesn't extend all the way to the LIC site, the topological strain created by the helicase enzymes involved in the creation of the single stranded DNA and migration of the Holliday junction may be enough to cause the non-covalently linked DNA molecules to dissociate. This dissociation would cause the loss of the complementary strand.

The integration/transformation frequency of linearized vectors is typically 10^2 transformants per μg DNA (Yamamoto, Moerschell, Wakem, Ferguson, & Sherman, 1992), although this efficiency is rarely reported and may vary based on transformation technique. The maximum amount of DNA used in this study was 1 μg. This amount of DNA could have produced positive colonies in a typical transformation of linearized DNA, however; the LIC step may complicate the cloning and lead to a lower efficiency. With more optimization, it is possible this procedure may generate positive clones.

Chapter 6: General Discussion and Conclusions

6.1 General discussion, conclusions, and recommendations for future research

Contamination by LAB during bioethanol production is a major concern of the bioethanol industry because it lowers productivity in an industry that already has narrow profit margins. In this study, our first approach to this problem was to engineer a yeast strain to secrete the bacteriocin leucocin A. While the presence of leucocin A activity was detectable in the yeast culture supernatant, not enough leucocin A was present to inhibit LAB in a cell-culture situation (Section 2.3.3, 2.3.7). Leucocin A activity was low despite using a highly active transcriptional promoter and using a bacteriocin that does not contain oxidizable methionine residues, which are two major theories for low levels of bacteriocin secretion proposed by other research groups (Basanta et al., 2009; Schoeman et al., 1999; Van Reenen et al., 2003). Investigations into the low activity indicate that leucocin A is likely interacting with the yeast cell membrane during the secretion process and the cell wall is preventing diffusion of the protein from the cells (Section 2.3.3, 2.3.4). There are also a number of other explanations that could account for poor performance of the system: self-aggregation of the bacteriocin within the media, proteolytic activity in the culture supernatant, and deactivation of the protein during the excretion process. Because these bacteriocins are hydrophobic in nature and their normal mechanism of action involves them embedding in the target cell membrane, it makes sense that they could interact with the cell membrane of yeast, and the experimental findings support this. The interaction between the secreted bacteriocin and the yeast cell membrane may be a difficult obstacle to overcome in order to succeed at creating a yeast strain that secretes an industrially-feasible amount of bacteriocin.

If secretion of the bacteriocin at industrially-feasible levels is achieved, one major remaining obstacle to this process would be balancing the metabolic burden of producing high levels of bacteriocin while maintaining high levels of ethanol production. This balancing act within the yeast cells could limit total ethanol production or slow ethanol production rates, so it would require further study and optimization. One way to encourage production of bacteriocin only in the event of microbial contamination could be to regulate the transcription of the bacteriocin gene using a promoter that is induced by the presence of lactic acid or lactic acid bacteria. Kawahata *et al.* (2006) performed microarray assays in the presence and absence of

lactic and acetic acids acids identified a promoter (for the gene *PDR12*) that is highly active in the presence of lactic, acetic, and hydrochloric acid. This promoter could be used to control the expression of a bacteriocin gene in order to mitigate the constant metabolic burden of expressing and secreting high levels of the protein and allow for the protein to be only secreted when needed.

It may also be possible to induce protein expression by coupling a LAB-detection system with an inexpensive means for induction. Lactic acid levels could be determined chemically by in-line high performance liquid chromatography. A quick, inexpensive process for induction of gene expression would be required. Due to the large volumes within a bioethanol reactor, a chemical induction method would likely be impractical due to cost and response time, so other methods would need to be sought. For example, light-inducible expression systems, which would be very low-cost, have been recently demonstrated (Hughes, Bolger, Tapadia, & Tucker, 2012), but would not work well in a highly turbid system like grain mash.

Even sub-optimal levels of bacteriocin secretion may be part of an overall strategy to control microbial contamination. As demonstrated in Chapter 4, many antimicrobials have additive properties when used in combination to inhibit microbes (Section 4.3.2). A bacteriocin-secreting strain could be used as part of an antimicrobial strategy that uses a number of antimicrobials such as hop compounds, conventional antibiotics, and additional measures discussed in Chapter 1, such as the addition of chitosan or urea-hydrogen peroxide. The antimicrobials chosen in this study were used because they are readily available for commercial use. Future research in this area should include expanding screening for additive and synergistic properties of several other antimicrobials that are not yet used commercially, but have the potential for commercial use. This could include other conventional antibiotics, chitosan, phage, and phage endolysin.

A finding in Chapter 4 that warrants further research, especially if leucocin A or other Class IIa bacteriocins are used in industrial systems, is the apparent synergy between leucocin A and nisin for several strains of LAB, as well as the apparent synergy between leucocin A and Isostab® for one LAB strain. It was difficult to determine and quantify the actual synergy in the system because leucocin alone, even at high loading doses, did not inhibit the LAB strains tested in liquid cultures, although it inhibited most of the strains in spot-on-lawn assays (Section 4.3.1). Although it did not inhibit any of these strains alone in liquid culture, it inhibited several of the

strains in combination with other inhibitors at concentrations of 0.5X of the inhibitor's MIC, indicating that there is likely a synergistic relationship. This synergistic relationship could be very advantageous if leucocin A is used with other inhibitors or bacteriocins.

This apparent synergy between leucocin A and nisin also emphasizes the importance of developing commercial means of production for other bacteriocins, such as the development of a methodology to produce carnocyclin A in Chapter 3. Although carnocyclin was not successfully cyclized in detectable quantities using the SICLOPPS methodology, the areas for further experimentation suggested in Section 3.4, such as using a different residue +1 to the I_C domain, and/or using a different or alternately located affinity tag may lead to more productive results. A high-throughput, synthetic biology approach could be taken to rapidly screen a larger number of alternative arrangements of different affinity tags (6His, chitin binding, etc.), the position of the affinity tag (N or C terminal), and the carnocyclin residue used as the residue +1 to the I_c domain. If cyclization is achieved, this methodology could be used to produce Class IV bacteriocins for industrial purposes such as food preservation and to control contamination in industry.

Synthetic biology approaches, like the one described above, require alternative cloning methodologies to quickly assemble large molecules, such as the methodologies described in Chapter 5. The LIC methodology performed in this study was only modestly successful in assembling plasmids, compared with results reported by others for methodologies such as yeast recombination cloning (Oldenburg et al., 1997), the Gibson assembly methodology (2009), the ligase-cycling reaction (Kok et al., 2014), or a methodology using 60 bp synthetic overlapping regions to guide recombination events (Kuijpers et al., 2013). Although the cloning success rate did not approach the 95% success rate reported by Kuijpers et al. (2013), this methodology may be useful in some instances.

The rise in interest in the development of products from renewable biological sources coupled with the rise of synthetic biology has led to the development of a large product portfolio from microorganisms. *S. cerevisiae* has been used to produce a vast array of products in addition to ethanol, and this list is ever-expanding. *S. cerevisiae* has recently been engineered to produce numerous other products, including itaconic acid (Blazeck et al., 2014), polymer monomers such as succinic and muconic acids (Borodina & Nielsen, 2014), triacetic acid lactone (Cardenas & Da Silva, 2014), all of which can be used to replace petroleum products.

Because of the industrial importance of *S. cerevisiae* the production of ethanol and its growing importance in the production of other chemicals from biological feedstocks, it is important to continue to develop mechanisms to protect fermentations from contamination by LAB. Despite the challenges, secretion of bacteriocins may be a solution to these contamination issues if it is optimized for use during fermentation processes. Secreting bacteriocins in combination with other inhibitors could be an excellent solution to LAB contamination.

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Appendix A: Gene sequences used in this study

```
> [MFA1 GFP LEU - 1080 bp] [DEFINITION]:  [LOCUS]: UA37-1.SEQ
ATGAGGTTCCCTTCAATTTTTACTGCCGTTCTTTTTGCTGCTTCATCTGCGCTAGCGGCACCTG
CAAACACAACTACGGAGGACGAGACTGCTCAGATCCCAGCTGAAGCTGTCATTGACTATAGTGA
TTTAGAAGGCGACTTTGACGCCGCTGCATTACCTTTATCAAATTCTACCAATAACGGACTAAGT
TCTACAAACACTACAATTGCTTCTATAGCCGCAAAAGAAGAAGGTGTCCAACTGGATAAACGTA
TGTCTAAGGGTGAAGAATTGTTTACTGGCGTAGTGCCCGTATTGGTCGAATTAGATGGTGATGT
CAACGGACAAAAATTCAGTGTGAGCGGGGAAGGTGAGGGTGATGCCACATATGGAAAGTTGACG
TTGAACTTCATATGTACTACAGGTAAACTTCCAGTTCCCTGGCCAACGCTAGTTACCACCTTTT
CGTATGGAGTCCAATGTTTTTCCCGTTATCCTGACCATATGAAGCAACATGACTTTTTCAAATC
AGCTATGCCAGAAGGTTATGTACAGGAAAGAACTATCTTTTACAAGGATGATGGGAATTATAAA
ACAAGAGCAGAAGTGAAATTTGAAGGGGATACCTTAGTTAACAGAATCGAATTGAAAGGTATTG
ATTTTAAAGAGGACGGTAATATTCTGGGCCATAAGATGGAATACAATTACAATTCACATAATGT
TTACATTATGGGTGATAAACCGAAAAATGGTATAAAAGTTAATTTCAAGATAAGACATAATATT
AAGGATGGTAGCGTTCAATTAGCAGATCACTATCAGCAAAATACTCCGATCGGCGATGGGCCTG
TATTGCTTCCAGATAACCATTACTTATCCACCCAATCCGCCCTAAGTAAGGATCCAAACGAGAA
GAGGGATCACATGATACTGCTTGAGTTCGTGACAGCTGCAAGAATTACACATGGTATGGACGAA
TTGTATAAGAAATACTACGGTAATGGTGTTCACTGTACTAAAAGCGGTTGCTCCGTAAACTGGG
GAGAAGCATTCTCTGCTGGCGTTCACAGGTTGGCCAATGGAGGAAATGGCTTTTGG

Features :
GFP     : [256 : 969]
MFA1    : [1 : 255]
LeuA    : [970 : 1080]
```

Figure A2-1 Gene sequence of SS_{MFA1}-GFP-LeuA ordered from BioBasic Inc. (Markham, ON, Canada). Sequence was codon-optimized for expression in *S. cerevisiae*.

> [MFA1-LEU-GFP - 1080 bp] [DEFINITION]: [LOCUS]: UA37-2.SEQ

ATGAGGTTTCCGTCCATTTTTACTGCAGTTCTATTCGCTGCATCATCGGCTCTTGCAGCTCCCG
CTAATACGACTACAGAAGATGAAACAGCACAAATCCCAGCTGAGGCCGTCATCGACTACAGCGA
CTTAGAGGGTGATTTTGATGCGGCCGCTTTGCCCTTATCAAATTCTACTAACAATGGTTTGAGT
AGCACCAATACCACTATCGCAAGTATTGCTGCAAAAGAAGAAGGGGTGCAACTGGATAAAAGGA
AATACTACGGCAACGGTGTTCACTGCACAAAATCTGGGTGTTCTGTGAATTGGGGTGAAGCCTT
TTCCGCAGGCGTGCACCGTTTGGCAAATGGAGGAAACGGTTTTTGGATGTCCAAAGGAGAAGAG
TTGTTCACTGGCGTTGTACCAGTTTTGGTAGAACTAGATGGTGATGTAAACGGTCAAAAATTTT
CCGTTAGTGGTGAGGGAGAAGGTGATGCCACCTACGGTAAACTTACTCTGAATTTCATATGTAC
CACAGGCAAATTGCCAGTTCCTTGGCCGACGTTGGTCACCACATTCTCTTATGGTGTCCAGTGT
TTCAGTAGATACCCTGATCACATGAAACAACATGATTTCTTTAAATCAGCGATGCCTGAAGGAT
ATGTGCAAGAAAGAACAATTTTCTATAAAGATGATGGTAATTATAAGACAAGAGCTGAAGTCAA
GTTTGAAGGGGATACTTTAGTAAACAGAATAGAATTAAAAGGGATAGATTTTAAGGAAGACGGC
AATATTCTTGGTCATAAGATGGAATACAACTATAATTCACATAATGTCTATATTATGGGTGACA
AACCAAAGAATGGAATCAAGGTTAACTTTAAGATTAGACATAACATTAAAGATGGCAGCGTTCA
ACTGGCCGACCATTACCAGCAAAATACCCCAATAGGTGATGGACCTGTTTTACTACCTGACAAT
CACTATTTATCTACGCAGTCAGCTCTGTCTAAGGACCCAAACGAGAAGCGTGACCATATGATAC
TTTTAGAATTTGTAACTGCCGCTAGGATTACTCATGGTATGGATGAGCTATATAAG

Features :

MFA1 : [1 : 255]

LeuA : [256 : 366]

GFP : [367 : 1080]

Figure A2-2 Gene sequence of SS_{MFA1}-LeuA-GFP ordered from BioBasic Inc. (Markham, ON, Canada). Sequence was codon-optimized for expression in *S. cerevisiae*.

```
> [MFA1-8AA-LEU - 393 bp]
ATGAGATTCCCATCCATTTTCACCGCCGTATTATTTGCTGCCTCCTCCGCCTTAGCTGCTCCTG
CCAACACTACAACCGAAGACGAAACTGCACAAATCCCAGCCGAAGCTGTTATTGATTATTCCGA
CTTGGAAGGTGACTTTGACGCTGCTGCTTTGCCTTTATCCAATAGTACCAATAACGGTTTATCT
TCAACTAACACTACAATTGCATCAATAGCTGCAAAAGAAGAAGGTGTACAATTGGATAAGAGAT
TCCCAACAGCCTTAGTTAGAAGAAAGTACTACGGTAACGGTGTACATTGTACCAAGTCTGGTTG
CTCAGTCAACTGGGGTGAAGCATTCTCAGCAGGTGTCCATAGATTGGCTAACGGTGGTAACGGT
TTCTGGTGA

Features :
LeuA    : [280 : 393]
8AA     : [256 : 279]
MFA1    : [1 : 255]
```

Figure A2-3 Gene sequence of SS$_{MFA1}$-8AA-LeuA ordered from GenScript (Piscataway, NJ, USA). Sequence was codon-optimized for expression in *S. cerevisiae*.

> [Split intein Carnocyclin A - 642 bp]

ATGGTTAAAGTTATCGGTCGTCGTTCCCTCGGAGTGCAAAGAATATTTGATATTGGTCTTCCCC
AAGACCATAATTTTCTGCTAGCCAATAGTCTAATTAACGCAGGTTTAACAGTAGGGTCTATTAT
TTCAATTTTGGGTGGGGTCACAGTCGGTTTATCAGGTGTCTTCACAGCAGTTAAAGCAGCAATT
GCTAAACAAGGAATAAAAAAAGCAATTCAATTATTAGTTGCATATGGTATCGCACAAGGTACAG
CTGAAAAGGTTGTATGCCTCAGTTTTGGCACCGAAATTTTAACCGTTGAGTACGGCCCATTGCC
CATTGGCAAAATTGTGAGTGAAGAAATTAATTGTTCTGTGTACAGTGTTGATCCAGAAGGGAGA
GTTTACACCCAGGCGATCGCCCAATGGCATGACCGGGGAGAGCAGGAAGTATTGGAATATGAAT
TGGAAGATGGTTCAGTAATCCGAGCTACCTCTGACCACCGCTTTTTAACCACCGATTATCAACT
GTTGGCGATCGAAGAAATTTTTGCTAGGCAACTGGACTTGTTGACTTTAGAAAATATTAAGCAA
ACTGAAGAAGCTCTTGACAACCATCGTCTTCCCTTTCCATTACTTGACGCTGGGACAATTAAAT
AA

Features:

Ic : [1 : 90]

In : [271 : 642]

Carnocyclin : [91 : 270]

Figure A3-1 Sequence of split intein carnocyclin A DNA obtained from Sigma. Sequence was codon-optimized for expression in *Escherichia coli*.

Appendix B: Protein Secondary structure of carnocyclin A

SLINAGLTVGSIISILGGVTVGLSGVFTAVKAAIAKQGIKKAIQLLVAYGIAQGTAEKVV

Figure B3-1 Primary structure of carnocyclin A with GS residue highlighted in yellow. LS residue is highlighted in green. α-helical regions of the protein secondary structure are underlined.

Appendix C: Checkerboard experimental details

Table C1-1 A checkerboard experiment. Wells of the plate are shown as cells surrounded by double lines. Various amounts of inhibitors X and Y corresponding to FIC_X and FIC_Y (see 4.2.5), respectively, were added to the microplate as shown resulting in 64 unique combinations of the inhibitors. A diluted microbial culture was then added to each of the wells and the plate was incubated at 30°C. Shaded cells represent wells where growth was observed. The number in each well represents the ΣFIC and was calculated by adding the corresponding FIC_X and FIC_Y. Wells with red ΣFICs displayed no growth and were positioned adjacent to a well where growth was observed. Thus, in this example, the minimum (ΣFIC_{min}) and maximum (ΣFIC_{max}) FICs are 0.50 and 1.03, respectively.

Inhibitor Y (FIC_Y)	Inhibitor X (FIC_X)							
	2.00	1.00	0.50	0.25	0.13	0.06	0.03	0.00
2.00	4.00	3.00	2.50	2.25	2.13	2.06	2.03	2.0
1.00	3.00	2.00	1.50	1.25	1.13	1.06	1.03	1.00
0.50	2.50	1.50	1.00	0.75	0.63	0.56	0.53	0.50
0.25	2.25	1.25	0.75	0.50	0.38	0.31	0.28	0.25
0.13	2.13	1.13	0.63	0.38	0.26	0.19	0.19	0.13
0.06	2.06	1.06	0.56	0.31	0.19	0.12	0.09	0.06
0.03	2.03	1.03	0.53	0.28	0.16	0.09	0.06	0.03
0.00	2.00	1.00	0.50	0.25	0.13	0.06	0.03	0.00

Appendix D – Protocol for cloning using LIC methodology

Vector preparation

1. Digest vector with *Nde*1 according to manufacturer's directions. Purify vector using a PCR purification kit or gel extraction kit.
2. React 37 fmol of vector in 5mM DTT, 2.5 mM dGTP, 1X T4 polymerase buffer (Novagen), and 4 U LIC Qualified T4 polymerase (Novagen) for 40 min at 22 °C, then 15 min at 75 °C.

Insert preparation

1. Amplify insert DNA with forward primer with the 5' sequence TCACTGATCTCATATG and the reverse primer with the 5' sequence GCCCTCACATTCCATATG.
2. Confirm PCR product size. Purify using a PCR purification kit or a gel extraction kit.
3. React 480 fmol insert in 5mM DTT, 2.5 mM dCTP, 1X T4 polymerase buffer (Novagen), and 4 U LIC Qualified T4 polymerase (Novagen) for 40 min at 22 °C, then 15 min at 75 °C.

Ligation independent cloning

1. Mix 2.5 µL of the prepared vector with 2.5 µL of the prepared insert.
2. Incubate at room temperature for 5 min.
3. Add 1.25 µL of 25 mM EDTA.
4. Incubate at room temperature for 5 min.
5. Transform into yeast cells using DMSO assisted yeast transformation.