University of Alberta

ISOLATION, CHARACTERIZATION AND GENETIC ANALYSIS OF COLICIN Y1O1, A NATURAL VARIANT OF COLICIN V

by

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DEDICATION

To my mother, Oc-hee Park

who waited so long for this with unconditional love, encouragement and trust

and

to my beloved wife Yun-Hee Regina Jung.

None of this would be possible without your love and sacrifice.

With all my love to

my happy, energetic, bright, boundless three sons,

Sung-Han, Dae-Han, Jang-Han.

All of you keep my spirit alive!

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LIST OF ABBREVIATIONS

AU	Activity units
ABC	ATP Binding Cassette
Bac^-	Bacteriocin negative
Bac^+	Bacteriocin positive
bp	Base pair(s)
ColV	Colicin V
ColY101	Colicin Y101
DEC	Diarrheagenic *Escherichia coli*
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *E. coli*
HC	hemorrhagic colitis
HPLC	High performance liquid chromatography
HUS	hemolytic uremic syndrome
kb	Kilo base(s)
kDa	Kilo Dalton
LAB	Lactic acid bacteria
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MFP	Membrane fusion protein
MW	Molecular weight
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
RBS	Ribosome binding site
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate

Sec	General secretion pathway
TFA	Trifluoroacetic acid
Vol/vol	Volume per volume
wt/vol	Weight per volume

CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

1.1 General Introduction

Bacteriocins are a diverse group of antibacterial proteins or peptides produced by bacteria. Bacteriocin synthesis occurs in both Gram-positive and Gram-negative bacteria, playing an apparent role in competitive interactions in a microbial community (Konisky, 1978; Pugsley, 1984a; Tagg *et al.*, 1976). The first "bacteriocin" that was discovered was produced by *Escherichia coli* and it was described as "principle V" by Andre Gratia in 1925. It was later given the name colicin V (Frick *et al.*, 1981). Since then, there has been major interest in other colicins produced by *E. coli* and a large number of colicins have been reported (Konisky, 1982). As a group, bacteriocins are defined as proteinaceous, antimicrobial compounds. The bacteriocins produced by Gram-negative bacteria are generally referred to as colicins or microcins. Some exceptions are the cloacins from *Enterobacter cloacae*; pesticins from *Yersinia pestis*; klebicins from *Klebsiella* species; and marcescins from *Serratia marcescens* (Lakey *et al.*, 1994). In addition to the colicins and related compounds of the *Enterobacteriaceae*, bacteriocins have been described for all genera of lactic acid bacteria (LAB) (DeVuyst and Vandamme, 1994; Klaenhammer, 1993; Tagg *et al.*, 1976). Bacteriocins have bactericidal or bacteriostatic activity generally against a narrow spectrum of closely related bacteria.

The producer strains protect themselves from the adverse effect of their own bacteriocins by producing an immunity protein. The gene for the immunity protein is closely linked to the gene for bacteriocin production. In some cases of colicins from Gram-negative bacteria and in many cases of class II bacteriocins of Gram-positive

bacteria, the bacteriocins are produced as prepeptides and they are converted into active bacteriocins in the process of export from the bacterial cell. Other bacteriocins, such as the lantibiotics of Gram-positive bacteria and microcins of Gram-negative bacteria, are synthesized as prepeptides and they undergo extensive posttranslational modification to generate the active compound (Baquero *et al.* 1978; Baquero and Moreno, 1984; Klaenhammer, 1993; Konisky, 1982; Nes *et al.*, 1996; Pugsley and Oudega, 1987). The colicins of *E. coli* and other closely related members of the family *Enterobacteriaceae* are the best characterized antibacterial peptides and proteins and they have been the focus of numerous research studies.

1.1.1 Definition and classification of colicins

With a few possible exceptions, the colicins are a large group of plasmid-encoded antimicrobial proteins or peptides that are synthesized by *E. coli* and other members of the Family *Enterobacteriaceae* under conditions of environmental stress, such as nutrient depletion or overcrowding. It is assumed that this provides a means for the producer strain to compete against other strains of closely related bacteria (James *et al.*, 1996). Microcins generally differ from colicins in size of the molecules, the degrees of post-translational modification and types of secretion mechanisms.

To date, more than 35 colicins have been described and at least 20 of them have been described in detail (Table 1.1). In general, colicins consist of a single polypeptide chain with molecular mass ranging from 29 to 75 kDa. They are not post-translationally modified and cell lysis is required for their release from the producer cell (Pugsley, 1984a,b). In contrast, microcins have a relatively small molecular size (less than 5 kDa), they undergo extensive post-translational modification to produce the active form of the microcin and they do not undergo cell lysis (Baquero and Moreno, 1984). The range of

Table 1. 1. Known colicins and their characteristics[a]

Colicin	Plasmid type[c]	Colicin receptor protein	Translocation System	Mode of action	Molecular weight[f] kDa	References
A	LMMa	BtuB/OmpF	TolA,B,Q,R	Pore formation	62.989*	Morlon *et al.*,. 1983 Lloubes *et al.*, 1984 Cavard *et al.*, 1985
B[b]	HMM	FepA	TonB, ExbB,D	Pore formation	54.732*	Schramm *et al.*, 1987
D[c]	LMMa	FepA	TonB, ExbB,D	16s rRNA cleavage	74.688*	Roos *et al.*, 1989
E1	LMMa	BtuB/TolC	TolA,Q,R	Pore formation	57.279*	Chan *et al.*, 1985
E2	LMMb	BtuB/OmpF	TolA,B,Q,R	DNAase	61.561*	Cole *et al.*, 1985
E3	LMMb	BtuB/OmpF	TolA,B,Q,R	16s rRNA cleavage	57.960*	Chak and James, 1984 Jakes and Zinder, 1974
E4	LMMb	BtuB/OmpF	TolA,B,Q,R	16s rRNA cleavage	-	Pugsley, 1983
E5	LMMb	BtuB/OmpF	TolA,B,Q,R	16s rRNA cleavage	-	Curtis *et al.*, 1989
E6	LMMb	BtuB/OmpF	TolA,B,Q,R	16s rRNA cleavage	58.011*	Akutsu *et al.*, 1989 Lau and Condie, 1989
E7	LMMb	BtuB/OmpF	TolA,B,Q,R	DNAase	61.349*	Males and Stocker, 1980 Chak *et al.*, 1991
E8	LMM	BtuB/OmpF	TolA,B,Q,R	DNAase	70 kDa**	Cooper and James, 1984 Toba *et al.*, 1988
E9	LMM	BtuB/OmpF	TolA,B,Q,R	DNAase	-	Cooper and James, 1984 James *et al.*, 1987
G[d]	LMM?	Fiu	TonB, ExbB,D	Membrane lysis?	5.5 kDa**	Bradley, 1991b
H[d]	HMM?	Fiu	TonB, ExbB,D	Membrane lysis?	100 kDa**	Bradley, 1991b
Ia[b]	HMM	Cir	TonB, ExbB,D	Pore formation	69.406*	Mankovich *et al.*, 1986
Ib[b]	HMM	Cir	TonB, ExbB,D	Pore formation	69.923*	Mankovich *et al.*, 1986
K	LMMa	Tsx/OmpF,A	TolA,B,Q,R	Pore formation	59.611*	Pilsl and Braun, 1995c
L[e]	-	?/OmpA	TolA,Q,?	Pore formation	45 kDa**	Hauduroy and Papavassiliou, 1962, Foulds and Shemin, 1969

Table 1.1. Continued

M[b]	HMM	FhuA	TonB, ExbB,D	Inhibition of murein synthesis	29.453*	Ölschläger and Braun, 1987
N	LMMa	OmpE,C PhoE	TolA,Q,R	Pore formation	41.696*	Pugsley, 1987
U	LMM	OmpA,F LPS	TolA,B,Q,R	Pore formation	66.289*	Šmajs *et al.*, 1997
5	LMM	Tsx/TolC	TonB, ExbB,D	Pore formation	53.137*	Bradley and Howard, 1992 Pilsl and Braun, 1995a,b
10	LMM	Tsx/TolC	TonB, ExbB,D	Pore formation	53.342*	Pilsl and Braun 1995a Viejo *et al.*, 1992
Bacteri-ocin 28b	-	?/OmpA,F, LPS	TolA,B,Q,R	Pore formation	47.462*	Viejo *et al.*, 1992
Pesticin I[b]	LMM	FyuA (*Y. pestis*)	TonB, ExbB,D	Hydrolysis of murein	40.043*	Ben-Gurion and Hertman, 1958, Pilsl *et al.*, 1996
Cloacin DF13	LMMa	IutA/OmpF	TolA,Q,R	16s rRNA cleavage	59.283*	Van den Elzen *et al.*, 1983
V[b*]	HMM	Cir	TonB, ExbB,D	Pore formation	8.735*	Gilson *et al.*, 1990

a: Table based on information from Pugsley (1984a) and Šmarda and Šmajs (1998)

b: Absence of lysis gene, b*: suggested microcin

c: Lysis gene identified but not characterized

d: appears to be plasmid-encoded but not characterized, not "SOS" inducible, gene for colicin G seems to be a deleted form of plasmid pColH

e: LMMa (type Ia), low-molecular-mass plasmid (less than 40 kb), multi-copy-number, amplifiable, not transferrable by conjugation (Tra^-): LMMb (type Ib), as LMMa but not amplifiable: HMM (type II), high-molecular-mass plasmid (50 to 200 kb), low-copy-number, non amplifiable, often Tra^+: -, plasmid not identified, appears to be chromosomally encoded.

f: *, calculated molecular weight based on protein and/or gene sequence, DNA sequences available from the literature: **, approximate molecular weight determined by SDS-PAGE

susceptible strains or species is generally narrow and limited to closely related bacterial species because of the specificity of the receptor of sensitive cells. A unique characteristic of colicins is that colicin synthesis is induced by "SOS" response, which leads to higher production during the exponential growth phase. The "SOS" response is an error-prone mechanism of repairing damaged DNA in *E. coli* by the coordinated induction of several enzymes. Damaged DNA activates an enzyme called RecA protease, and the protease cleaves a protein called LexA repressor. Many genes involved in repair functions become activated when this repressor is cleaved. This system is often the cause of mutations following either chemical or UV mutagenesis. In contrast to the general colicins, microcins are usually synthesized during the stationary phase and their synthesis is not "SOS" inducible (Kolter and Moreno, 1992). Producer strains are protected by a constitutively-produced immunity protein against the homologous colicin or microcin but there is no cross-immunity to heterologous types of colicins and microcins (Baquero and Moreno, 1984; Pugsley, 1985). Depending on the presence of colicin-specific immunity and receptor proteins, insensitivity of the cells to a particular type of colicin can be the result of: (i) immunity, (ii) resistance or (iii) tolerance. Colicin-resistant bacteria either have an immunity protein or lack a particular receptor, whereas tolerant bacteria can adsorb the colicin molecules but they have a mechanism that blocks the translocation of the colicin into the cell (Lazdunski and Cavard, 1982; Pugsley, 1984b, 1985).

To kill the target cells, colicins interact with susceptible cells in three consecutive steps: (i) binding to a specific receptor on the outer membrane, (ii) translocation through the host's cell membrane, and (iii) killing action on the specific intracellular target (Bénédetti and Geli, 1996; Braun *et al.*, 1994; Konisky, 1982; Lakey *et al.*, 1994). According to the classical scheme for differentiation of the colicins, they are classified into groups on the basis of their receptor specificity, modes of action, method of translocation and the absence of cross-immunity between producer strains (Luria and

Suit, 1987; Pugsley 1984a,b). Colicins in group A include A, E1 to E9, K, L, N and U. They utilize the Tol multi-protein complex to penetrate the cells. This consists of six proteins: TolA, TolB, TolQ, TolR, PAL and Orf2 (Davies and Reeves, 1975a; Lazdunski, 1995; Vianney *et al.,* 1996; Webster, 1991). Colicins in group B include B, Ia, Ib, D, M, 5, and 10 and they utilize TonB and its associated proteins ExbB and ExbD to penetrate the cells (Braun, 1995; Davies and Reeves, 1975b; Lazdunski *et al.*, 1998; Pilsl and Braun 1995a,b,c; Postle, 1993) (Table 1.1).

A difference in modes of action of different types of colicins is another important aspect of colicin classification. Colicins can be divided into four groups depending on how they act on susceptible cells. The two major groups are the pore-forming and enzymatic groups of colicins. The pore-formers are the largest group of colicins that include: ColA, B, E1, Ia, Ib, K, L, N, U, V, 5 and 10. They are translocated into the outer surface of the cytoplasmic membrane and form a cytoplasmic ion channel that results in depolarizing of the membrane and reduction of the membrane potential (Lakey *et al.,* 1994; Parker *et al.*, 1989; Qui *et al.*, 1996). Enzymatic colicins share many characteristics with the pore-formers except that their lethal activity is due to non-specific DNA degradation or 16s rRNA cleavage. Colicins E2, E7-E9 are known to cause nonspecific degradation of DNA (Šmarda *et al.,* 1990). Colicins E3, E4 and E6 kill the cells by cleavage of 16s rRNA that results in the inhibition of protein synthesis (Lasater *et al.*, 1989). Activity of two other colicins is confused with that of the pore-formers. They include inhibition of peptidoglycan synthesis resulting in cell lysis by colicin M (Harkness and Ölschläger, 1991; Köck *et al.*, 1987) and disruption of the membrane potential of the target cells by colicin V (Gilson *et al.*, 1990). Colicin M is the only colicin known to inhibit biosynthesis of both peptidoglycan and O-antigen. Autolysis of susceptible cells by colicin M occurs as a secondary effect following inhibition of peptidoglycan synthesis (Harkness and Ölschläger, 1991). It shares common features

with other colicins in terms of "SOS" induciblity, a three functional domain structure, receptor binding and translocation mechanisms (Köck *et al.*, 1987). This colicin is unusual among the colicins that have been studied and it has a distinguishable smaller molecular size of 29 kDa. The molecular weight of colicins generally ranges between 42 and 75 kDa.

Colicin V disrupts the membrane potential of the target cells but does not form channels (Gilson *et al.*, 1990). Unlike other colicins, colicin V has unique characteristics. It is the smallest known colicin with a molecular weight of 8,735 Da and its synthesis is not "SOS" inducible. Neither lysis protein nor a signal sequence of the *sec*-pathway is required for its release. Instead, it has its own dedicated export system, similar to that of the bacteriocins of Gram-positive bacteria. Because of these atypical features, it is considered that colicin V might belong to the family of microcins (Asensio *et al.*, 1976; Fath *et al.*, 1992; Kolter and Moreno, 1992), but it kept its original name for historical reasons. However, the characteristics of colicin V do not fit the general characteristics of microcins. Colicin V has more in common with so-called class II bacteriocins from LAB (Fath *et al.*, 1994; Håvarstein *et al.*, 1995; Nes *et al.*, 1996).

1.1.2 Occurrence of colicins

The colicinogenic trait is common among the enteric bacteria. Colicin-positive strains can be isolated at high frequencies among natural and clinical isolates of *E. coli*. This indicates that colicinogenic bacteria may have a selective advantage against other strains of the same species. Although there is a wide range of frequencies of colicinogenic strains in different test populations, the data can also be affected by the test methods and indicator strains that are used. Data for frequency of colicinogeny range from 25 to 50% (Hardy, 1975; Riley and Gordon, 1992). Little is known about the factors

that determine the frequency of colicinogeny in natural populations. It has been suggested in several studies that the colicin trait is more common among pathogenic than among commensal isolates and that it may be more prevalent among isolates from humans than animals (Selander *et al.*, 1987).

Over 30% of lactose-fermenting, Gram-negative isolates from the River Seine were colicin-positive (Pugsley, 1984a). Riley and Gordon (1992) evaluated colicin production of *E. coli* isolated from the *E. coli* reference collection (ECOR). The data revealed that 50% of isolates from humans and 16% of isolates from animals were colicin-positive. From other studies, the incidence of colicinogeny among *E. coli* O157:H7 was 33 to 50 % and the almost universal production of colicin D was reported (Frost *et al.*, 1989; Scotland *et al.*, 1987; Whittam *et al.*, 1993). Similar estimates were obtained from the study by Bradley (1991a), in which 34% of serotype O111:- strains produced colicin. However, no connection between colicinogeny and pathogenicity was demonstrated and colicin itself seems not to contribute to virulence (Bradley *et al.*, 1991a; Quakenbush and Falkow, 1979; Waters and Crosa, 1991).

1.1.3 Colicin synthesis and its genes

Colicins are encoded on either type 1 or type 2 Col plasmids but never by both plasmids. This represents a fundamental difference in the way in which colicins are produced. Colcin L is an exception because it is chromosomally-encoded. Type 1 Col plasmids are small, they have a multiple copy number and they are not conjugally transferred (tra^-); while type 2 Col plasmids are large, they have a low copy number and they are often conjugative (tra^+). Depending on amplifiability, type 1 plasmids are differentiated into two subgroups: type 1a that replicate in the absence of continued protein synthesis by the host (amplifiable); and type 1b that is not amplifiable (Pugsley,

1984a; Pugsley and Oudega, 1987).

With the exception of colicin V, and possibly colicins G and H, synthesis of colicins is regulated by the so-called "SOS" regulatory response mechanism that is normally associated with the control of genes involved in the system for the repair of damaged DNA. Under normal conditions, the *lexA* repressor gene switches off the Col plasmid so that very few cells in the population produce colicin (Lakey *et al.*, 1994). This repressor is inactivated by the action of the RecA protease in response to DNA damage. As a result, colicin synthesis is switched on. Colicin production in a growing population can be induced by mutagenic agents such as U.V. light or mitomycin C. The LexA repressor binding regions are found slightly upstream of the colicin structural gene. The "SOS" system normally exhibits very tight repression and it ensures that only a small population of cells undergoes colicin expression and export (Lakey *et al.*, 1994).

Colicin plasmids also encode one or two other types of proteins: an immunity protein and a protein that is the so-called lysis protein that is required for colcin release from the cell. All colicin-producing organisms encode immunity proteins that confer specific protection of the producer strain against the colicin of the producer strain but not against heterologous colicins. For the nuclease colicins, the immunity gene is oriented in the same direction as the structural and lysis genes and transcription is proportional to these genes. For pore-forming colicins, the immunity gene is oriented in the opposite direction to the structural and lysis genes and it is constitutively transcribed. The subsequent export of colicins depends on the expression of the lysis gene, which is located downstream of the colicin gene and under control of the colicin gene promoter. This gene is only present in type 1, small plasmids (Mankovich *et al.*, 1984; Schramm *et al.*, 1987). The lysis protein is a small lipoprotein that is responsible for easier release from the cells. Information on the immunity and lysis proteins is described separately (Fig. 1.1).

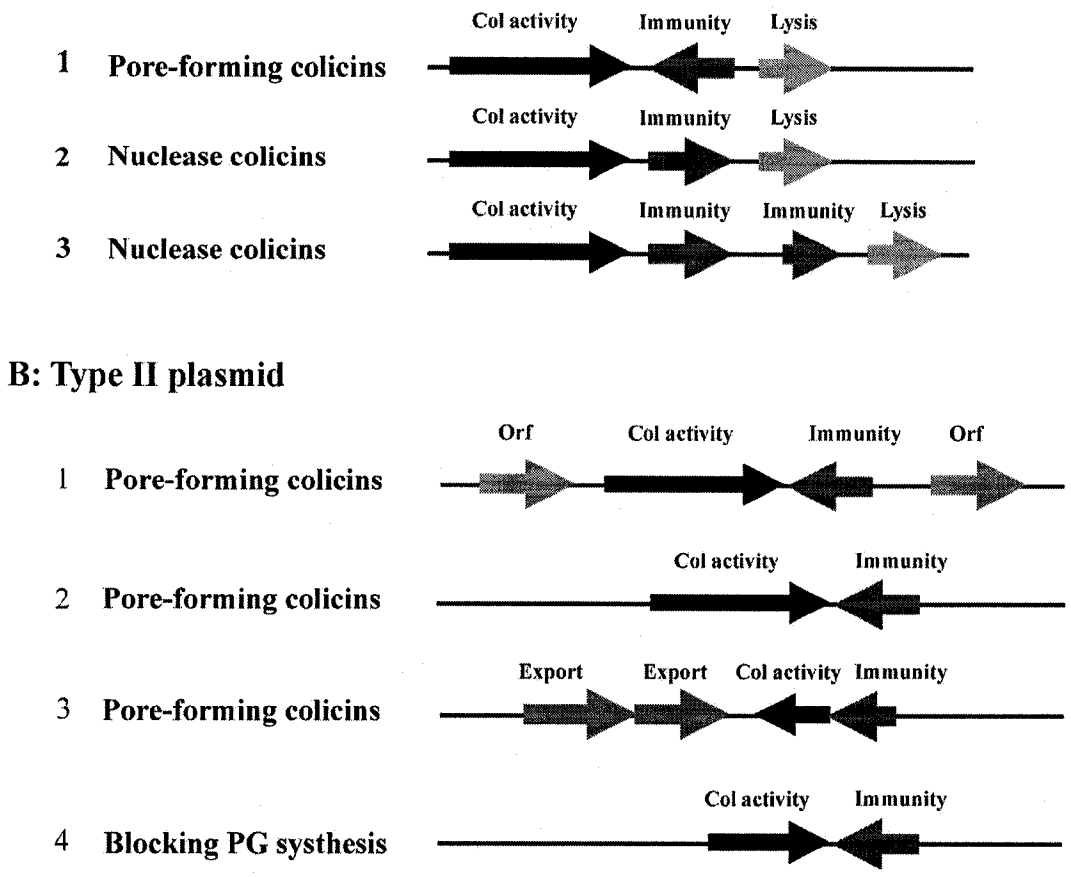

Figure 1.1 Arrangement of the colicin structural, immunity and lysis genes or export genes (see text for details).

A: colicin gene cluster encoded in type I plasmids, the immunity gene for pore-forming colicins is in the opposite direction to structural and lysis genes (1) and is in the same direction for nuclease colicins (2). An additional colicin E8 immunity gene is known to be encoded in the case of colicins E3 and E6 (3).

B: colicin gene cluster encoded in type II plasmids, No lysis gene was identified. Orientation of immunity gene for colicins Ia, Ib (1), B (2), and M (4) is in the opposite direction to the structural gene. Colicin V (3) atypically has two genes that encode export proteins. Based on data from Fath *et al.* (1992) and Riley and Gordon (1996).

1.1.4 Export of colicins

Many proteins of Gram-negative bacteria are synthesized in the cytoplasm and they are exported to the periplasm, the outer membrane, or the extracellular medium. In most cases, signal sequences mediate protein release and translocation across the cytoplasmic membrane. The N-terminal extensions of cytoplasmic proteins in Gram-negative bacteria possess special signal sequences that are cleaved during the export process. The signal sequence makes the proteins recognizable by the specialized proteins of the export machinery, in particular the general secretion pathway (Pugsley, 1993).

Colicins are released into the extracellular environment by quite different systems than other protein secretion systems found in *E. coli* (Lakey *et al.*, 1994). Unlike other proteins of Gram-negative bacteria, for many colicins there are no N-terminal signal sequences or export-related domains in the colicin molecules (Braun *et al.*, 1994). Export of colicins across the cytoplasmic membrane, is either regulated by a semi-specific or non-specific process and it is accompanied by the non-specific release of cytoplasmic and periplasmic molecules (Baty *et al.*, 1987; Lakey *et al.*, 1994).

The export of colicins depends on the presence of the so-called lysis gene, whose expression is required for efficient release of colicin from the cell. The gene for the lysis protein is located downstream of the colicin gene and its expression only occurs following induction of colicin synthesis, because its regulation is under control of the colicin gene promoter (Cavard *et al.*, 1985; Cavard, 1992). The lysis protein is initially transcribed as a precursor protein containing a signal sequence and it is post-translationally modified by removal of the N-terminal signal peptide and by the attachment of a fatty acid to the newly exposed N-terminal cysteine residue that is acylated. Another function that it may have is to activate the host-cell phospholipase in the outer membrane resulting in the production of lysophospholipids that destabilize the

membrane structure sufficient to permit increased transmembrane diffusion of colicin and some other proteins (Cavard *et al.*, 1987; Pugsley, 1984a; Pugsley and Schwartz, 1984) and to allow small solutes to leak out of the cell in the absence of Mg^{++} (Baty *et al.*, 1987). Mature lysis proteins are found in both the outer and inner membranes (Howard *et al.*, 1989; Van der Wal *et al.*, 1995) except for the lysis protein of colicin N that is only found on the outer membrane (Pugsley, 1988). Pugsley (1988) showed that the precursor and mature forms of the colicin A lysis protein and unusually stable signal peptide that is cleaved from the precursor protein, are all associated with the inner membrane, while the mature form appears much later in the outer membrane. The stability of the signal sequence after cleavage may contribute to the release of colicin as shown for cloacin DF13 (Luirink *et al.*, 1991).

The semi-selective export of colicin encoded by type I Col plasmids is facilitated by the lysis protein, simultaneously causing cell lysis and death of producer strains with concomitant leaking of some other proteins and small solutes. Therefore, the producer strains should minimize the amount of colicins that they produce once committed to the sacrificial process of colicin production and export. This may explain why colicin is produced in a much larger amount than the lysis protein, particularly if a critical concentration of lysis protein is required to trigger colicin export (Pugsley, 1984a). However, the synthesis and export of colicins B, Ia, Ib and M encoded by type II Col plasmids is less clear. Although synthesis of colicin from type II Col plasmids is also induced by "SOS" regulation, colicins in this group are produced in smaller amount than colicins encoded by type I plasmids, mainly because of their low copy number. Because type II Col plasmids do not contain the lysis gene, these colicins are less efficiently released even when the level of production is increased by increasing the number of colicin genes. Although the export mechanism for these colicins remains unclear, their production at a lower concentration seems to be due to their accumulation in the

cytoplasm causing membrane damage and cell death (Pugsley, 1984a)

1.1.5 Interaction of colicin with susceptible bacteria

The interaction between colicin molecules and their receptor proteins in the outer membrane is highly specific resulting in a narrow antibacterial effect. The lethal action of colicins on sensitive cells generally proceeds through a three-stage mechanism: (1) receptor binding; (2) translocation across the outer membrane protein and/or periplasmic space; and (3) lethal interaction with an intracellular target (Lakey *et al.*, 1994; Pugsley, 1984a,b). This mechanism is strongly related to the three distinct functional domain structure of colicins. Colicins are taken into the cytoplasm by binding to a specific receptor protein in the outer membrane (Braun *et al.*, 1994). The central domain of the colicin molecule is responsible for binding to the specific receptors located at the surface of target cells. The N-terminal domain is important for translocation of colicins across the cell envelope interacting with two different uptake pathways: the Ton and Tol systems. The C-terminal domain of colicins determines the type of lethal activity (Bénédetti and Geli, 1996; Cramer *et al.*, 1995). Because each functional domain of the colicin molecule is known to be relatively independent, it is possible to construct a hybrid molecule from colicin and other types of colicins or bacteriocins. Kageyama *et al.* (1996) showed that the receptor and translocation domains of pyocin could be incorporated into a functional colicin E3.

Once colicins are released from the producer cells, they reach the target cells by diffusion and attach to specific receptors on the surface of susceptible cells. These receptors are critical factors in defining the activity spectrum of the colicin and they also serve as transporters of other important nutrients for the bacterial cells. The same receptor protein may be used by different colicins, but the actual domain of the receptor

that attaches to the different colicins may be the same or different. Binding of colicins to its receptor is an energy independent protein-protein interaction (Pugsley, 1984a). The currently known colicins and their receptors are listed in Table 1.1.

Colicin translocation through the cell envelope can occur by the Ton and Tol multi-protein complex systems. Depending on how the colicins interact with either Ton or Tol translocation system (Fig 1.2), colicins have been separated into groups A or B (Davies and Reeves, 1975a,b). Group A colicins include colicins A, E1-E9, K, L, N, and U and they require the Tol protein complex to be translocated into cells. The four membrane proteins TolA, B, Q and R are involved the formation of a bridge between the inner and outer cell membranes connected to a passive diffusion channel such as OmpF. An additional peptidoglycan lipoprotein, PAL, may also play a role to connect or stabilize this complex. Among these, TolA plays a central role in this energy-independent pathway. TolA is anchored to the cytoplasmic membrane by its N-terminus and its central domain spans the periplasmic space. The C-terminus of TolA interacts with receptors on the outer membrane, binding the N-terminal domain of group A colicins. Interaction of group A colicins with the TolA protein depends on a putative TolA box that has a consensus pentapeptide sequence DG(T/S)G(S/W) located in its N-terminus (Pilsl and Braun, 1995a). TolQ and R are located on the cytoplasmic membrane (Fig. 1.2) (Lazdunski, 1995; Vianney *et al.*, 1996; Webster, 1991).

In contrast, the Ton translocation system for group B colicins (colicin B, D, Ia, Ib, M, 5 and 10) has a high affinity, energy-dependence and it is formed by a TonB-ExbB-ExbD complex (Braun, 1995; Postle, 1993). This protein complex facilitates the flow of energy from the cytoplasm to the outer membrane for the energy–dependent transport of ferric siderophore complexes, vitamin B12, as well as group B colicins. The TonB system is believed to serve as a mechanical linkage between the inner and outer membranes. This energized conformation of TonB opens channels in the outer membrane formed by

FepA and BtuB receptor proteins. Subsequently, TonB is reduced to its lower energy state and therefore needs to re-energize after each opening event (Fig. 1.2). Similar to the relationship between group A colicins and the TolA system, Group B colicins also possess a highly conserved region called the TonB box (GIMAV) in their N-terminal domain that is also found in the FepA and BtuB receptors (Bell *et al.*, 1990; Schramm *et al.*, 1987). Interestingly, there is a high degree of similarity between the TonB and TolA systems. The TolA protein is analogous to the TonB protein system. TolQ and R share significant homology and topologies with ExbB and D, respectively, and they are functionally interchangeable, exhibiting cross-reactivity (Fig. 1.2).

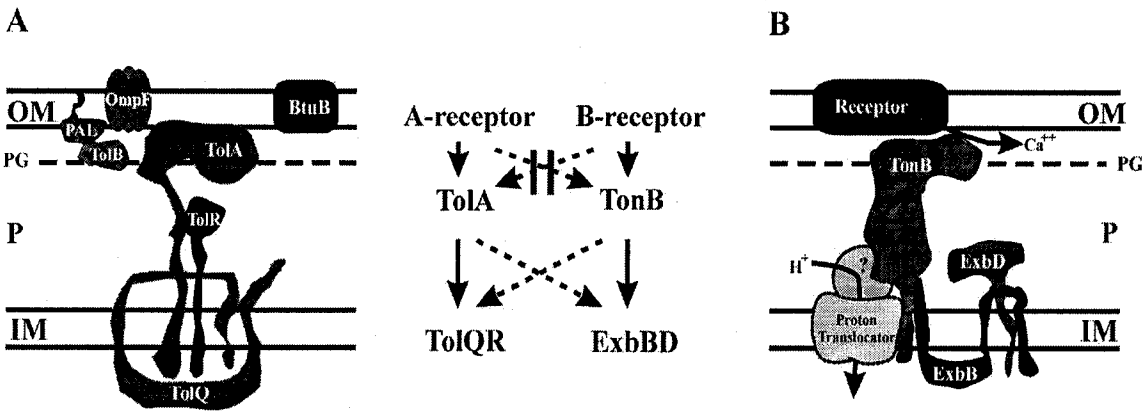

Figure 1.2 The colicin translocation model of (A) Tol system for group A colicins and (B) Ton system for group B colicins. TolQ and TolR have 75% sequence similarity and share topological homology with ExbB and ExbD, respectively. TolQR and ExbBD exhibit cross-reactivity with TonB and TolA proteins, respectively. In contrast, no sequence similarity was found in TolA and TonB except in the regions inserted in the cytoplasmic membrane, which has functional importance. TolA and TonB require receptor specificity and cannot replace each other's functions. See Lazdunski *et al.* (1998) for further details. OM, outer membrane; IM, inner membrane; P, periplasm; PG, peptidoglycan. Figure adapted from Lazdunski *et al.* (1998) and Wiener *et al.* (1997).

Finally, as the colicins reach their target, they debilitate the target cells in several different ways. The C-terminal domain of colicins carries the different types of lethal activity. The largest group of colicins (colicin A, E1, N,B, Ia, Ib, K, U, 5 and 10) form ion channels in the cytoplasmic membrane, resulting in the depolarization of the membrane. The second most common group comprises those colicins that are known to degrade DNA or to inhibit protein synthesis by cleavage of 16s rRNA. Colicin M has a unique mode of action. It causes lysis of susceptible cells by inhibition of peptidoglycan biosynthesis (Braun *et al.*, 1974; Harkness and Ölschläger, 1991; Schaller *et al.*, 1982). Colicin V differs from other known colicins listed in Table 1.1. More detailed information on colicin V will be presented in a separate section.

1.1.6 Colicin immunity

Killing sensitive bacteria with colicins is highly efficient and it occurs by specific mechanisms. Colicins make their way into susceptible cells by first binding to specific outer membrane receptors and they enter the periplasm by either Ton or Tol pathways, each of which has a specific connection with the receptor and is composed of two or more periplasmic or inner-membrane proteins. Once the colicin is in the periplasm, colicins exert the lethal action to their designated targets. Cells become insensitive to colicins in three different ways. Resistant cells are protected against the colicins by the absence or alteration of a functional receptor(s). In contrast, colicin tolerant cells have a receptor but they do not have a functional colicin translocation system or the translocation pathway after binding to the receptor is blocked. Regardless of the presence of functional receptors and translocation pathways, the most pronounced safeguard for cells against a colicin is the immunity protein that specifically interacts and inactivates an invading colicin. Immunity proteins are integral inner membrane proteins that are

laterally diffused to ensure rapid recognition of colicins that are entering the cell (Geli and Lazdunski, 1992; Goldman *et al.*, 1985).

Colicin-producing *E. coli* protect themselves from the suicidal effect of their own colicin by co-synthesis of a dedicated immunity protein (Geli *et al.*, 1986; Koniski, 1978). The function of the immunity protein does not involve an alteration in the colicin receptor and it occurs independent of the colicin translocation system. The specific nature of the interaction of the immunity protein with the relevant colicin allows a more precise differentiation among colicins that have the same receptor and import pathway because producer strains are resistant to their own colicin but sensitive to other colicins, even from the same cytotoxic class of bacteriocins (Pugsley and Oudega, 1987).

Immunity proteins that are specific for enzymatic colicins are transcribed with the colicin in a 1:1 ratio because the gene for the immunity protein is located in the same operon. The immunity protein is bound to the colicin in the producing cell and it is released into extracellular medium with the colicin as an equimolar heterodimeric complex. Therefore, the producer strains are protected from exogenous and endogenous colicins. As the colicins commence their passage into a target cell, the immunity protein is separated from the colicin but when and where this occurs is not known (Kleanthous *et al.*, 1998). In contrast, immunity proteins for pore-forming colicins are not synthesized nor released as a 1:1 complex. Genes encoding immunity proteins for this group are transcribed from constitutive promoters in the opposite direction to the colicin structural gene. The pore-forming colicins are released as monomers in the free form and the immunity proteins cannot be detected in the culture medium. Immunity proteins for pore-forming colicins can be classified into two groups according to their respective sequence homologies (Schramm *et al.*, 1988). A-type immunity proteins include those for colicins A, B, N, and U. E1-type immunity proteins include those for colicins E1, Ia, Ib, K, 5 and 10 (Pilsl and Braun, 1995a,b). The same types of immunity proteins have similarities in

their amino acid sequence and topology. For example, the A-type immunity proteins for colicins A and B share 38% identity and 39% conservative substitutions, but the topology of the immunity proteins for colicins A and E1 (Cai and Cei, respectively) are markedly different. Cai has four transmembrane regions facing its N- and C-terminus in the cytoplasm (Geli *et al.*, 1989), while Cei crosses the cytoplasmic membrane three times extending its N-terminus into the cytoplasm and the C-terminus in the periplasm (see Fig. 1. 3) (Song and Cramer, 1991).

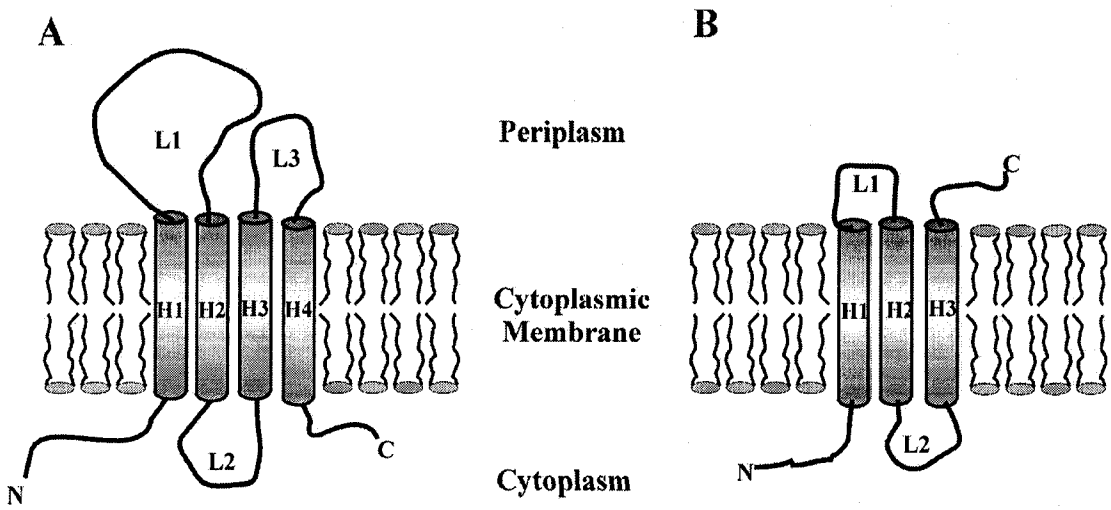

Figure 1. 3 Topology of the immunity protein of pore-forming colicins. A: A-type, B: E1-type. H and L denote the transmembrane α-helices and loops in cytoplasm and periplasm, respectively. C, C-terminus; N, N-terminus.

In the classical view of immunity protein specificity, colicin-producing cells only show resistance to their own colicin. However, it has been demonstrated that over-expressing immunity proteins or raising the intracellular immunity protein concentration could offer some protection against non-cognate colicins (Wallis *et al.*, 1995a). Non-

cognate cross-reactivity has been observed with immunity proteins for other pore-forming colicins. Geli and Lazdunski (1992) found that with high-copy-number plasmids colicin A immunity protein provides producer strains with partial protection against colicin B. In the case of nuclease colicins, non-cognate cross reactivity among E2, E8, and E9 has been reported (Kleanthous *et al.*, 1998; Wallis *et al.*, 1995b).

1. 2. Colicin V

Colcin V was first described by Gratia in 1925 and named by Frederiq in 1945. Although research on colicin V has been hampered by the low production and difficulty in purification, it has been genetically characterized and its secretion mechanism has been extensively studied. Colicin V displays several unique characteristics that distinguish it from other colicins. It has a considerably smaller molecular size than other colicins, its synthesis is not "SOS" inducible and it does not utilize a lysis protein for its release (Gilson *et al.*, 1990). Colicin V is exported by a signal sequence independent pathway. A dedicated export protein complex is required for its secretion. Because of these unique characteristics of colicin V, it was frequently considered that colicin V belongs to the family of microcins (Baquero *et al.*, 1978; Fath *et al.*, 1992)

However, the common characteristics of colicin V do not fit most of the general criteria of microcins. Microcins are much smaller than colicin V (less than 5 kDa) and they are post-translationally modified into an active form of mature peptide before being exported from the cell, whereas colicin V is not post-translationally modified. The role of the N-terminal leader peptide of microcins does not resemble that of colicin V. The leader sequence of colicin V plays a role in export by a dedicated translocation system. This is not the case for microcins. For microcin B17, the processing of the N-terminal sequence occurs prior to export of the microcin from the cell (Rodriguez-Sainz *et al.*, 1990; Yorgey

et al., 1992). The removal of the leader peptide from microcin J25 also occurs before posttranslational modification. The leader peptides of microcins B17 and J25 may play an important role in their enzymatic modification (Blond *et al.*, 1999; Madison *et al.*, 1997). On the other hand, Microcin C7, the smallest known microcin, does not contain a N-terminal leader peptide so it is directly synthesized and modified without a processing protein (Gonzalez-Pastor *et al.*, 1995).

Colicin V is considered to be more comparable to the class II bacteriocins of lactic acid bacteria than the other colicins or microcins of *E. coli* because of the similarity of its export mechanism and of the role of its leader peptide (Håvarstein *et al.,* 1995; Nes *et al.,* 1996). Microcin 24 from *E. coli* was also referred to as a "class II-type" bacteriocin (O'Brien and Mahanty, 1993). Among the recently discovered and genetically characterized microcins, microcin H47 (Azpiroz *et al.,* 2001; Rodriguez *et al.*, 1999) and microcin E492 (Lagos *et al.*, 1999; Pons *et al.*, 2002) were reported to possess strong relatedness in structure and export mechanism to colicin V. Both of these so-called microcins can be classified into the same group as colicin V; however, further characterization of Microcins H47, E492 and 24 is required to establish specific criteria for these bacteriocins. Colicin V is described below as a representative of this atypical group of colicins.

1.2.1 Colicin V and its genes

Colicin V (ColV) is encoded on a low-copy-number plasmid. Colicin V plasmids have been found in many strains of *E. coli* and they display considerable diversity range in size from 80 to 180 kb. These plasmids are often considered to be associated with virulence properties, because they have been found primarily among virulent enteric bacteria; however, there is no clear evidence of relatedness between colicin V and

virulence determinants except for the iron uptake system of aerobactin (for detailed information, see Waters and Crosa, 1991 and Williams and Warner, 1980).

A 900 bp fragment of the pColV-B188 plasmid was shown to include the colicin V structural and immunity genes (Frick *et al.,* 1981); however, this cloned fragment did not confer production of extracellular, active colicin V. Subsequently, colicin V and its immunity gene were localized at approximately the same region of the plasmid pColV-K30. The complete nucleotide sequence of a 4496 bp fragment that included this region revealed the genetic determinants for synthesis, immunity, and export of ColV. Four linked genes are contained in two converging operons: one with the *cvaC* structural gene and the *cvi* immunity gene; and the other containing two export genes, *cvaA* and *cvaB* (Fig. 1.4) (Gilson *et al.*, 1987, 1990).

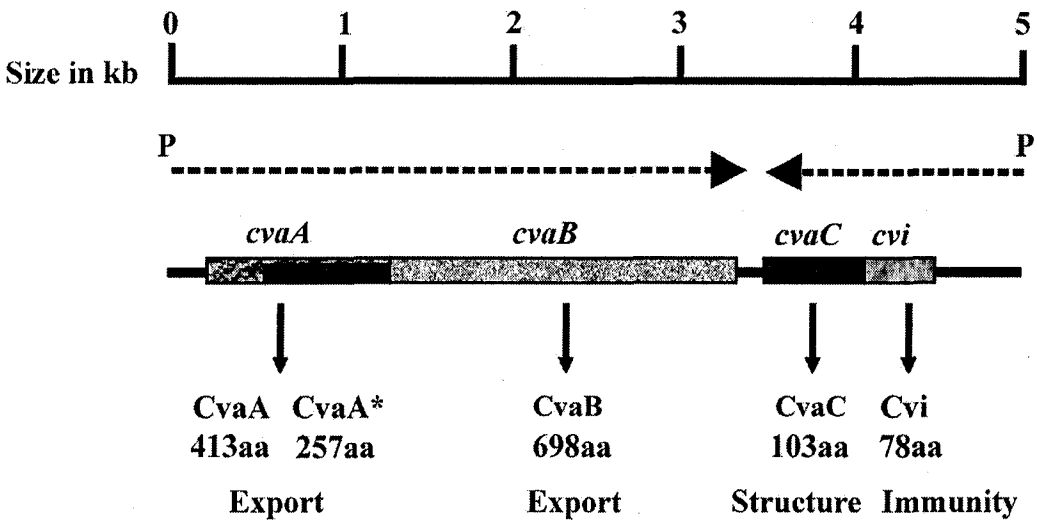

Figure 1. 4 The colicin V gene cluster from plasmid pColV-K30 and its size and function of the four colicin V gene products (adapted from Fath *et al.*, 1992 and information from Gilson *et al.*, 1987, 1990)

The *cvaC* gene is located directly downstream of *cvi* and encodes the 103 amino acids of the ColV precursor (pre-ColV). Pre-ColV contains a 15-amino-acid N-terminal leader peptide that has significant similarity to double-glycine-type leader peptides found in class II bacteriocins of lactic acid bacteria (Fath *et al.*, 1994; Håvarstein *et al.*, 1994). Pre-ColV is biologically inactive because of the N-terminal leader peptide that serves as a recognition signal for the transport system (Van Belkum *et al.*, 1996). The leader peptide is removed following a conserved Gly (-2), Gly (-1) site concomitant with extracellular secretion of the active ColV. For this reason it is referred to as a "double glycine type" leader peptide. The leader peptides in this group are presented in Table 1.2. The different double-glycine-type leader peptides also possess several consensus sequences: L(-12), S(-11), X(-10), X(-9), E(-8), L(-7), X(-6), X(-5), I(-4), X(-3), G(-2), and G(-1), and the glycine at the –2 position is considered to be 100% conserved (Håvarstein *et al.*, 1994). The resulting 88-amino-acid active ColV has a calculated mass of 8735.8 Da and it inhibits growth by disruption of the membrane potential of target cells (Yang and Konisky, 1984).

The *cvi* gene encodes a 78-amino-acid membrane protein that confers immunity to the cells. The C-terminus of the *cvi* gene overlaps the N-terminus of the ColV structural gene. The export of ColV is mediated by three linked membrane proteins that form a complex composed of CvaA, CvaB and TolC. The transport proteins CvaA and CvaB are encoded in the *cvaAB* operon and they are known to form a dedicated export system. The third membrane protein TolC is chromosomally encoded. Uptake of ColV by susceptible cells is primarily dependent on the presence of an outer membrane ColV receptor protein, Cir or Cir-like proteins (Davies and Reeves, 1975a,b), and mediated by the TonB-ExbB-ExbD import system that is also utilized by group B colicins. The schematic model for ColV production, export and import is shown in Fig. 1.5.

Table 1.2 Sequence alignment of double-glycine-type leader peptide of colicin V compared with class II bacteriocins of lactic acid bacteria and a *Brochothrix* sp.

Class II Bacteriocin[a,b]	Double-glycine-type leader peptide sequence	Reference
Colicin V[a]	MRTLTLNELDSVSGG A..	Gilson *et al.*, 1990
Consensus[b]	LS--EL--I-GG X..	Håvarstein *et al.*, 1994
	#--#*-**#--#---	
Brochocin A[c]	MHKVKKLNNQELQQIVGG Y..	McCormick *et al.*, 1998
Brochocin B[c]	MKKELLNKNEMSRIIGG K..	McCormick *et al.*, 1998
Carnobacteriocin A	MNNVKELSIKEMQQINGG D..	Worobo *et al.*, 1994
Carnobacteriocin B2	MNSVKELNVKEMKQLHGG V..	Quadri *et al.*, 1994
Carnobacteriocin BM1	MKSVKELNKKEMQQINGG A..	Quadri *et al.*, 1994
Carnobacteriocin X	MKSVKELNVKEMQQTIGG W..	Quadri *et al.*, 1997
Carnobacteriocin Y	MNKEFKSLNEVEMKKINGG S..	Quadri *et al.*, 1997
Enterocin A	MKHLKILSIKETQLIYGG T..	Aymerich *et al.*, 1996
Lactacin A[c]	MKQFNYLSHKDLAVVVGG R..	Fremaux *et al.*, 1993
Lactacin X[c]	MKLNDKELSKIVGG N..	Fremaux *et al.*, 1993
Lactococcin A	MKNQLNFNIVSDEELSEANGG K..	Holo *et al.*, 1991
Lactococcin B	MKNQLNFNIVSDEELAEANGG S..	van Belkum *et al.*, 1991
Lactococcin Gα[c]	MKELSEKELRECVGG G..	Håvarstein *et al.*, 1995
Lactococcin Gβ[c]	MKNNNNFFKGMEIIEDQELVSITGG K..	Håvarstein *et al.*, 1995
Lactococcin M[c]	MKNQLNFEILSDEELQGINGG I..	van Belkum *et al.*, 1991
Lactococcin N[c]	MKKDEANTFKEYSSSFAIVTDEELENINGS G..	van Belkum *et al.*, 1991
Leucocin A	MMNMKPTESYEQLDNSALEQVVGG K..	Hastings *et al.*, 1991
Leucocin B	MNNMKSADNYQQLDNNALEOVVGG K..	Hastings *et al.*, 1994
Mesentericin Y105	MTNMKSVEAYQQLDNQNLKKVVGG K..	Héchard *et al.*, 1993
Sakacin A	MNNVKELSMTELQTITGG A..	Axelsson and Holck, 1995
Sakacin P	MEKFIELSLKEVTAITGG K..	Tichaczek *et al.*, 1994
Pediocin PA-1	MKKIEKLTEKEMANIIGG K..	Marugg *et al.*, 1992
Plantaricin E	MMLQFEKLQYSRLPQKKLAKISGG F..	Diep *et al.*, 1996
Plantaricin F	MKKFLVLRDRELNAISGG V..	Diep *et al.*, 1996
Plantaricin J	MTVNKMIKDLDVVDAFAPISNNKLNGVVGG G..	Diep *et al.*, 1996
Plantaricin K	MKIKLTVLNEFEELTADAEKNISGG R..	Diep *et al.*, 1996

a: colicin V is produced by *E. coli*, and shows a double glycine cleavage motif
b: #, hydrophobic residue; *, hydrophilic residue
c: two-component bacteriocin

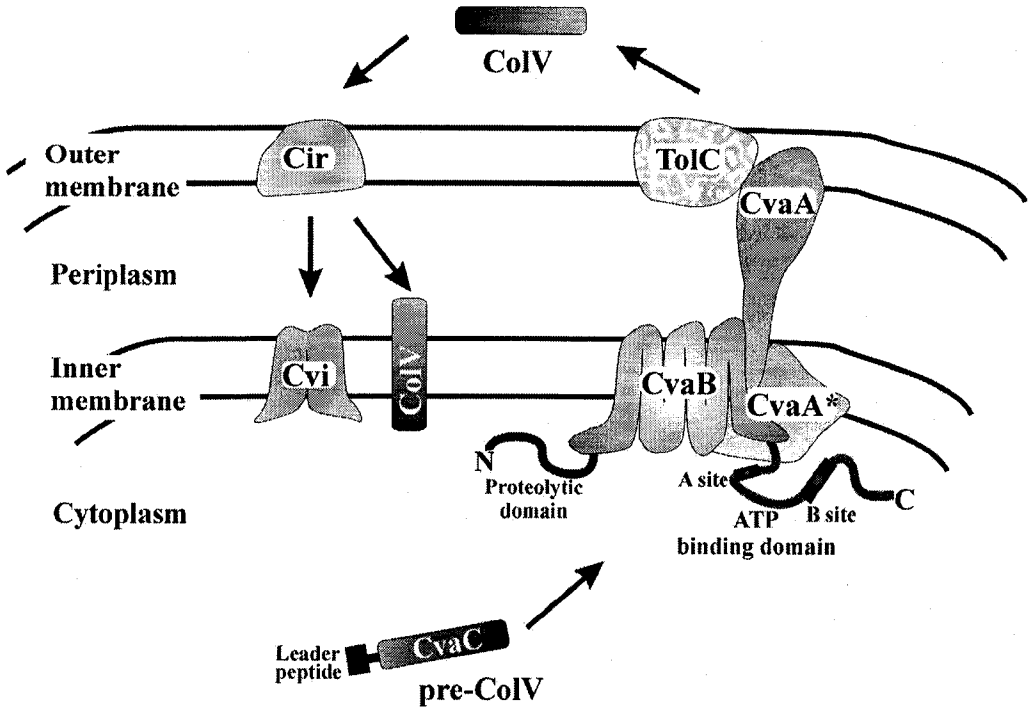

Figure 1.5 Proposed topological model for colicin V synthesis, export and import. The schematic model is based on data from Fath *et al.* (1992), Hwang *et al.* (1997) and Zhang *et al.* (1995).

1.2.2 The colicin V immunity protein

The ColV immunity protein Cvi is encoded by the *cvi* gene that is located immediately upstream of the structural gene for ColV and it is comprised of 78 amino acids with a molecular weight of 9.1 kDa. The ColV immunity protein is necessary and sufficient to fully protect the producer cells from the killing action of ColV (Fath *et al.*, 1992; Gilson *et al.*, 1990). Cells containing only the *cvi* gene in the presence of the

receptor Cir protein are fully immune to ColV, while cells without Cvi, regardless of expression of the export genes *cvaAB*, do not confer immunity to the cells (Zhang *et al.*, 1995)

Nucleotide sequence analysis revealed that the Cvi protein has two hydrophobic stretches in the N-terminal region that could constitute membrane-spanning domains. Cvi is membrane bound and it could only be detected in the cell lysate by purification. There is no predicted N-terminal signal sequence of Cvi, so it is likely that Cvi is localized in the cytoplasmic membrane by free diffusion after synthesis (Fath *et al.*, 1992; Gilson *et al.*, 1990).

1.2.3. Dedicated export system for colicin V

Unlike most colicins, the export of colicin V does not utilize a lysis protein for selective leakage nor does it undergo signal sequence dependent *sec*-pathway excretion from the cell. The extracellular translocation of ColV is mediated by two dedicated export proteins, CvaA and CvaB, encoded by the *cvaAB* operon with the support of the chromosomally encoded outer membrane protein TolC (Fig 1.5). The linked genes *cvaA* and *cvaB* are located directly adjacent to the *cvi/cvaC* operon and they are transcribed under the control of a single promoter. Similar to *cvi* and *cvaC*, these two open reading frames overlap suggesting translational coupling of this operon (Fath *et al.*, 1992; Gilson *et al.*, 1990). This dedicated export apparatus is sometimes referred to as a member of the multidrug-resistance (MDR)-like family of transporters because of the approximately 200-amino-acid putative ATP-binding domain that shares a high degree of similarity with a family of proteins involved in various transport processes (Higgins *et al.*, 1986). Proteins transported by this family of dedicated export proteins include: *E. coli* α-hemolysin, *Erwinia* protease, yeast a-factor and *Bordetella* cyclocin in which a

remarkable conservation of overall size and hydropathy profile are found. This subfamily is also referred to as the ATP-Binding Cassette (ABC) transporter superfamily. They transport a wide range of substrates such as inorganic ions, sugars, amino acids, peptides and proteins across the cytoplasmic membrane in prokaryotes and eukaryotes (Fath and Kolter 1993; Higgins, 1992).

The primary export protein CvaB in the ColV export system comprises 698 amino acids with a C-terminal cytoplasmic region that contains a highly conserved, 200-amino-acid typical ATP-binding cassette (Gilson *et al.*, 1990). This ATP-binding domain provides the energy of ATP hydrolysis to facilitate the secretion of ColV. From the amino acid and hydropathy analyses, it is predicted that CvaB possesses six potential transmembrane domains similar to other ABC transporters (Fig. 1.5). It was proposed that CvaB contains an N-terminal domain located in the cytoplasm as commonly found in the ABC transporters of other bacteriocins. This domain may function as a protease and it is responsible for removal of the double-glycine-type leader peptide from the precolicin V concomitant with secretion from the cell (Håvarstein *et al.*, 1995).

In addition to CvaB, the dedicated colicin V transport system requires an accessory protein, CvaA that is designated the membrane fusion protein (MFP). Proteins in the MFP family are similar in size and in hydropathy pattern to similar proteins in Gram-negative bacteria. They possess two characteristic hydrophobic regions: one in the N-terminus that spans the inner membrane, and the other near the C-terminus that is highly conserved and proposed to interact with the outer membrane (Dinh *et al.*, 1994; Wandersman and Delepelaire, 1990). It is suggested that this group of proteins functions to connect the inner and the outer membranes thus facilitating the passage of substrates out of the cell. However, the role of MFPs is not clearly understood (Skvirsky *et al.*, 1995). CvaA is anchored in the inner membrane by its N-terminal hydrophobic domain. Its C-terminus may connect CvaB with outer membrane protein TolC. It was shown that

cvaA gene produces two in-frame products, CvaA and CvaA*, estimated to be 43 and 27 kDa, respectively (Gilson *et al.*, 1990). CvaA* is a truncated form of CvaA that lacks the hydrophobic residues found in the N-terminal region of CvaA and, thus, was supposed to be located in the cytoplasm. CvaA* is not required but it enhances the secretion of ColV by stabilizing CvaA (Hwang *et al.*, 1997).

1.2.4 The general protein secretion pathway

The transport of bacterial proteins to their destination mostly involves the signal-sequence-dependent general secretion pathway (*sec*-pathway). In Gram-negative bacteria, such as *E. coli*, this pathway requires at least five products of *sec* genes and the proteins synthesized as a precursor contain signal peptides in their N-termini. The signal peptide plays a role as a targeting signal, and is cleaved concomitant with translocation of the protein across the membrane by the signal peptidase (translocase). Most proteins exported by this system are localized in the periplasm or the outer membrane (Pugsley, 1993; Randall and Hardy, 1995; Wickner *et al.*, 1991). Signal peptides have three domains that are structurally similar. The N-terminal domain is a short, polar region. The H-domain is composed of predominantly hydrophobic residues and alanine in the center region. The C-terminal domain is usually polar and contains signal sequences that are recognized by the signal peptidase. Even though there are no known colicins or microcins that use this system for their secretion, some bacteriocins from lactic acid bacteria that are translocated by the *sec*-pathway have been described (Eijsink *et al.*, 2002; Nes *et al.*, 1996; Worobo *et al.*, 1995). In addition, Zhang *et al.* (1995) showed that colicin V could be exported to the periplasm by the *sec*-dependent pathway with the OmpA signal sequence replaced. McCormick *et al.* (1999) reported that replacement of the leader peptide of ColV with the signal peptide of divergicin A allowed export of

active ColV by the general secretion pathway. Heterologous expression of colicins by the general secretion pathway is one of the important parts of this study. Therefore, this system is reviewed further in this section.

The general secretion system of bacteria is a multi-subunit protein complex, which is comprised of at least five integral membrane subunits SecY, SecE, SecG, SecD, SecF and the dissociable peripheral subunit SecA. Another component, SecB, a cytoplasmic chaperone protein, is also involved in this process targeting the preprotein to the membrane. SecB binds to the preprotein and prevents native folding and aggregation (Kumamoto, 1991; Lacker *et al.*, 1990). It targets the preprotein for export by virtue of the direct affinity of SecB for the SecA subunit of translocase (Wickner and Leonard, 1996). SecA is a dimeric protein located in the cytoplasm and each subunit has two nucleotide binding domains. It can bind functionally to the integral membrane complex, SecYEGDF. This binding activates SecA both as a receptor for the SecB-protein complex and as an enzyme for the binding and hydrolysis of ATP, and its insertion into and partially across the membrane (Economou and Wickner, 1994). Membrane insertion of SecA allows the signal peptide domain of the preprotein to bind SecY and SecE, and then, consecutive process stimulates the ATPase activity of SecA. The energy from two consecutive ATP hydrolyses drives the release of the preprotein from SecA and promotes release of SecA from the cytoplasmic membrane (Den Blaauwen and Driessen, 1996). During this process, two other integral membrane proteins, SecD and SecF, are believed to play an important part in protein secretion in the later stage involved with preprotein release from the translocation channel. At the last step, the signal peptidase cleaves the signal peptide to release the mature protein (Pugsley, 1993).

In Gram-positive bacteria, the secY gene of *L. lactis* has been cloned and sequenced (Koivula *et al.*, 1991) indicating that the *sec*-pathway is also used for protein release in lactic acid bacteria and that it is homologous to the *sec* system of other bacteria.

One example of these proteins or peptides that are produced by lactic acid bacteria and secreted via this pathway is divergicin A, a bacteriocin produced by *C. divergens* LV13 (Worobo *et al.*, 1995). The signal peptide from this bacteriocin will be used in this research for the study of heterologous expression of colicin. The schematic representation of general secretion pathway is shown in Fig. 1.6.

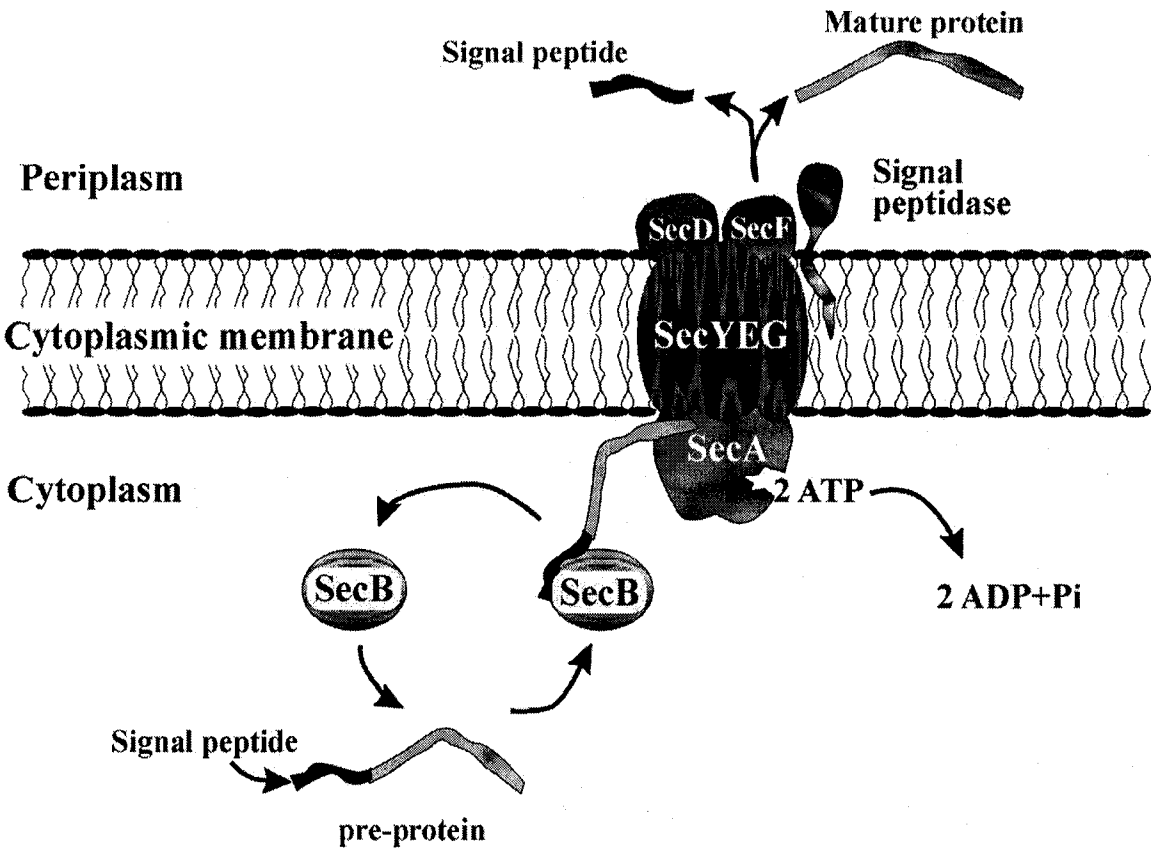

Figure 1.6 Schematic overview of the general protein secretion pathway in *Escherichia coli* (see text for details). Figure adapted from Wickner and Leonard (1996) based on data from Pugsley (1993).

1.2.5 Functional complementation of colicin V export

As mentioned before, many bacterial proteins that lack a typical N-terminal signal sequence are secreted by dedicated export systems. Generally, the bacterial ABC transporter requires at least one additional component, the so-called accessory protein. An additional outer membrane protein such as TolC is also required in Gram-negative bacteria due to the presence of the additional barrier posed by the outer membrane (Fath *et al.*, 1991). Among ABC export systems, the dedicated exporters of α-hemolysin (HlyBD) in *E. coli* and *Erwinia* protease (PrtDEF) are well characterized and share common features with the colicin V transporter (CvaAB). The ABC transporter CvaB and MFP protein CvaA in the ColV system is structurally and functionally homologous to HlyB, PrtD and HlyD, PrtE, respectively. The export of colicin V and α-hemolysin requires an outer membrane protein, TolC. PrtF in the *Erwinia* protease transport system is homologous to TolC. In addition, Fath *et al.* (1991) determined that, among these three export systems, Hly and Prt export systems were able to functionally complement CvaAB and export active colicin V from *E. coli*. The individual Hly export proteins were also able to functionally substitute for their respective Cva homologs, albeit at a lower efficiency.

It is noteworthy that ColV is synthesized as a precursor containing double-glycine-type N-terminal leader peptide, which was previously only found in Gram-positive bacteria. Most class II bacteriocins from lactic acid bacteria are produced as precursors with an N-terminal leader peptide. The leader peptides normally consist of 15 to 24 amino acids and have a conserved double glycine processing site at position –2 and –1 that is removed during the secretion process (Håvarstein *et al.*, 1995). In contrast to the C-terminal secretion signal of α-hemolysin (Kenny *et al.*, 1991) and the *Erwinia* protease (Delepelaire and Wandersman, 1990), the N-terminal leaders of ColV and other

bacteriocins function as an export signal for secretion. Furthermore, it was shown that the ABC transport proteins of this group of bacteriocins contain an additional N-terminal domain that has leader peptidase activity. This domain is not present in other ABC transporters. Because of these unique characteristics, it was suggested that colcin V could be regarded as a member of class II bacteriocins of lactic acid bacteria (Håvarstein *et al.*, 1994, 1995).

Due to the fact that the double-glycine-type N-terminal leaders share high sequence similarity with a conserved $Gly^{-2}Gly^{-1}Xaa^{+1}$ processing site, it is assumed that a dedicated export system is present for class II bacteriocins and these class II bacteriocins and colicin V are structurally homologous. Functional complementation of leader peptides of the double-glycine-type associated with ABC transporters was investigated. Van Belkum *et al.* (1996) showed that ColV could be exported by *L. lactis,* albeit with a decreased level of production, using the replaced leucocin A leader peptide and the lactococcin A dedicated export machinery. Several other reports have described the production of bacteriocin via heterologous ABC transporters in other species (Allison *et al.*, 1995a,b; Van Belkum and Stiles, 1995). The leader peptides of leucocin A, lactococcin A and colicin V direct the secretion of divergicin A, a bacteriocin that is secreted by the signal sequence dependent general secretion pathway (Van Belkum *et al.*, 1996). In contrast, it was shown that the divergicin A signal peptide could also direct the expression of bacteriocins that undergo the signal independent dedicated export system such as carnobacteriocin B2 (McCormick, 1996), enterocin B (Franz, 1997) and colicin V (McCormick *et al.* 1999).

1.3 Colicins G and H

Colicins G and H were identified in 1948 and regrouped as colicin P in 1953 by Fredericq (1957). Since then, characterization of these colicins has been hampered by several factors including their instability, very low level of production, difficulty in purification, and rare occurrence. Hence, the information on colicins G and H is limited.

Colicins G and H are produced by *E. coli* strains CA46 and CA58, respectively, but the source of the isolates was not clear. According to Bradley (1991b), the host strains CA46 and CA58 were serotyped as O169:NM and O30:NM, respectively. They are non-pathogenic *E. coli* that are typically associated with humans. It has been suggested that ColG is closely related to ColH (Hughes *et al.*, 1978). Colicins G and H were classified as group B colicins that use the Ton-dependent uptake pathway and they use the same receptor protein Fiu (Davies and Reeves, 1975a,b). Both colicins showed almost identical resistance patterns against other colicins and their antibacterial spectra were shown to be the same. The approximate molecular size of colicins G and H were determined to be 5.5 and 100 kDa, respectively, by SDS-PAGE and ultrafiltration methods. Attempts to raise antisera in rabbits to establish that colicins G and H are identical were unsuccessful and curing of the wild-type host strains with novobiocin to identify the origin of the plasmid failed (Bradley *et al.*, 1991b).

There are several complications associated with the information on the genetic determinants of colicins G and H. It was suggested that the plasmid encoding colicin G might have arisen by extensive deletion of colicin H (Hughes *et al.*, 1978). Lehrbach and Broda (1984) found that pColG hybridized to a single 6.3 kb *Eco*RI portion of pColH but the plasmids pColG and pColH were very different in size (4.6 and 94 kb, respectively). This supported the hypothesis that pColG was a deleted form of pColH. Although plasmid pColG has been tentatively identified, transfer of colicinogeny and immunity

from the original producer strain to *E. coli* K12 was not achieved. Identity of a Col plasmid could not be confirmed from wild-type *E. coli* host strains of colicin G (Pugsley and Oudega, 1987). In addition to the facts mentioned above, several interesting aspects of colicin G were sufficiently useful to trigger the study of its potential for application in foods. The producer strain of colicin G was not associated with pathogenic strains of *E. coli* of human origin. The isolated plasmid pColG may be 4.6 kb (Lehrbach and Broda, 1984) or 5.0 kb (Bradley, 1991b) and its molecular weight was determined to be approximately 5.5 kDa, which is unusually small in size compared with other colicins (Bradley, 1991b). Moreover, it has been shown that its synthesis was not induced by "SOS" response (Pugsley and Oudega, 1987). The producer strain was resistant to almost every known colicin (Bradley, 1991b). More interestingly, the antibacterial activity spectrum of colicin G was shown to be broad and strong especially against diarrheagenic *Escherichia coli* (DEC) strains. The colicin G producer, CA48, killed all of the strains in a sample of 20 *E. coli* O157:H7 isolates (Bradley, 1991a) and 27 of 27 DEC strains tested (Murinda *et al.*, 1996).

1.4 Microcins

Microcins are a group of bacteriocins produced by members of *Enterobacteriaceae* that inhibit *E. coli* and closely related strains. Unlike most colicins, microcins are low-molecular-weight antimicrobial peptides (less than 10 kDa). Their synthesis is non-lethal for the producing cells and it is not mediated by the "SOS" repair pathway. Because of their small size, they are insensitive to some proteases and they are thermoresistant. Most microcins are synthesized on ribosomes as peptide precursors and the precursors subsequently undergo posttranslational modification to generate active moieties before being exported. The extent of the research on the different microcins is

variable: some have been extensively studied, while others remain relatively uncharacterized (Asensio *et al.*, 1976; Baquero and Moreno, 1984).

The genetic determinants of most microcins are encoded on plasmids. However, both microcin H47 and E492 are chromosomally encoded (Laviña and Gaggero, 1992; Wilkens *et al.*, 1997). Microcins have a wide range of cellular targets and they can be classified into several groups based on their mode of action. For example, type A microcins inhibit metabolic enzymes, type B microcins prevent DNA replication, type C microcins inhibit protein synthesis and type D microcins impair the cell's energy generating system (Liu, 1994). Eight different microcins have been characterized, namely microcin B17, C7, D93, E492, H17, J25, L, and 24 (Braun *et al.*, 2002; Garzón *et al.*, 2002; Pons *et al.*, 2002; Sable *et al.*, 2000). It has also been suggested that the microcins can be classified into two distinct groups based on features involved in their structure and translational modification.

Microcin B17, C7, D93, and J25 are included in class I. They are small molecules less than 5 kDa that undergo extensive posttranslational modification, and they have a cyclic structure. All of these microcins have specific intracellular targets: microcin B17 (MccB17) is reported to be an inhibitor of DNA gyrase (Vizen *et al.*, 1991); and microcin C7 (MccC7) exerts its bacteriostatic action by blocking protein synthesis (Guijarro *et al.*, 1995). Microcins B17 and C7 are the best known and their structure, modes of action and the genes involved in their biosynthesis, export, and immunity have been described in detail.

MccB17 is a glycine-rich peptide consisting of 43 amino acids; 26 of which are glycine (Bayer *et al.*, 1995; Roy *et al.*, 1998). The genes necessary for its synthesis, export and immunity are in an operon that consists of *mcbA-G*. The maturation pathway of MccB17 consists of at least three steps (Yorgey *et al.*, 1993). First, the product of *mcbA*, pre-MccB17, is synthesized as a 69-amino-acid prepeptide that includes a 26-

amino-acid N-terminal extension. Second, pre-MccB17 is post-translationally modified into proMccB17. Third, the 26-amino-acid leader peptide is cleaved by an unknown protease, resulting in active MccB17 by the action of MccB17 synthetase that is encoded by *mcbBCD*, which generates two thiazoles, two oxazoles and two 4,2-fused bis-heterocycles (Fig. 1.7). The export of MccB17 is mediated by the products of *mcbEF* that is an ABC-type transporter system, but interaction with leader peptide of MccB17 appears to be unlikely as shown for ColV and other class II bacteriocins of lactic acid bacteria (Garrido *et al.*, 1988; Yorgey *et al.*, 1993). The proteins from *mcbG* cooperate with proteins of *mcbEF* to provide immunity for the producer strains.

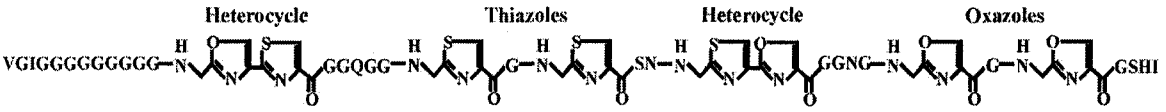

Figure 1. 7 Structure of microcin B17 after posttranslational modification and its sequence alignment. Figure adapted from Belshaw *et al.* (1998)

Microcin C7 (MccC7) is a linear heptapeptide containing modification at both the N- and C-termini (Garcia-Bustos *et al.*, 1984). The genetic determinants for MccC7 production are contained in 5 kb of the plasmid pMccC7 and there are 4 subregions that are genetically defined as α, β, γ, and δ that are transcribed in the same orientation. Two of these regions, β and δ confer immunity to the producer strains. The unmodified heptapeptide of MccC7 (MccA), MRTGNAD is encoded by a 21-nucleotide gene (*mccA*)

adjacent to the α subregion (Gonzalez-Pastor *et al.*, 1994). The gene *mccA* and subregions α to δ are transcribed in the same direction beginning with *mccA* and extending to δ (Novoa *et al.*, 1986). The genes in regions α and γ seem to encode the products required for modification and/or for the export of MccC7. Because MccC7 does not contain a leader peptide, no processing protein is required; however, it is known that the N-terminus undergoes acetylation and that its C-terminal modification remains unknown (Garcia-Bustos *et al.*, 1985). MccC7 inhibits protein synthesis of target bacteria.

Microcin J25 (MccJ25) is a highly hydrophobic cyclic peptide consisting of 21 unmodified amino acid residues. Genetic studies identified three genes, *mcjABC*, which are essential to its production and one gene, *mcjD*, which appears to help with export and conferring immunity. The *mcjBCD* genes are transcribed in the same direction, while *mcjA* is transcribed in the opposite direction. From the analysis of the gene sequence, it was revealed that *mcjA* encodes a peptide of 58 amino acids as a precursor containing a 37-amino-acid N-terminal extension (Solbiati *et al.*, 1999). This leader peptide is removed during peptide maturation that occurs with cyclization of the head and tail linkage of the 21-amino-acid prepeptide. The functions of McjB and C are not clearly defined but they appear to mediate MccJ25 biogenesis, which implies that they are involved in the cleavage of the leader peptide and the linkage of the pre-MccJ25 that results in the cyclic structure of the 21-amino-acid mature MccJ25 (Blond *et al.*, 1999).

Microcins E492, H47, L and 24 are class II microcins. They share several common properties with class IIa bacteriocins of lactic acid bacteria. Unlike class I microcins, they consist of unmodified amino acids and they contain double-glycine-type leader peptides that are involved in secretion that is mediated by the ABC transporter system. The target of antibacterial activity is known to be the bacterial membrane (Pons *et al.*, 2002). In this regard, both microcins H47 and E492 have many features in common with colicin V. Unlike other colicins and colicin V, microcins H47 and E492 are

chromosomally encoded.

Genetic studies of microcin H47 (MccH47) revealed that its genetic system comprises seven genes; four genes (*mchABCD*) for synthesis, two genes (*mchEF*) for secretion and a gene (*mchI*) for immunity. The unlinked *tolC* gene is required for export of MccH47 (Azpiroz *et al.*, 2001; Gaggero *et al.*, 1993; Rodriguez and Lavina, 1998). It was suggested that the structural gene *mchB* encoded a 75-amino-acid peptide precursor, which contains a double-glycine cleavage site in its N-terminus. This cleavage site is present in many prebacteriocins of lactic acid bacteria and colicin V of *E. coli*, which are secreted by a dedicated ABC transporter system (Table 1.3). The cleavage of its 15-amino-acid N-terminal leader peptide releases a microcin that contains 60 amino acid residues. It is exported from the cell by a dedicated ABC transport system (Rodriguez *et al.*, 1999). However, sequence analysis also revealed a *sec*-signal peptide at its N-terminus. Therefore, the possibility exists that there may be both types of export pathways present in the cell. However, it is not clear whether the 15 N-terminus amino acids are cleaved or the precursor MccH47 could possess activity of the same specificity as mature microcin. The exporter proteins of MccH47, MchE and MchF revealed 98% and 89% identity with CvaA and CvaB of colicin V, respectively, indicating that MchF is an ABC protein and MchE is a member of the MFP family. Furthermore, these two proteins are expressed in the same direction and have a small overlap, identical to that found between CvaA and CvaB in the ColV gene cluster (Azpiroz *et al.*, 2001).

Microcin E492 (MccE492) produced by *Klebsiella pneumoniae* is an unmodified microcin that is also structurally related to colicin V. MccE492 shares common characteristics of a double-glycine-type N-terminal leader peptide as in the case of MccH47 (Lagos *et al.*, 1999). The nucleotide sequence of 3.0 kb *Cla*I fragment obtained from pJAM434, which originated from *K. pneumoniae* RYC492, indicated that there are two overlapping ORFs that are responsible for encoding MccE492 structural (*mccA*) and

immunity (*mccB*) proteins. Lagos *et al.* (1999) explained that the structural gene, *mccA*, encodes a 99 or 103 amino acid precursor protein as the primary translation product with two methionines as the possible start codon at positions 1 and 5. Depending on either start codon, the first 15- or 19-amino-acid N-terminal region would be removed and the processed microcin E492 would be an 84-amino-acid molecule with a molecular weight of 7,887 Da. The cleaved 15- or 19-amino-acid N-terminus corresponds to a double-glycine-type leader peptide similar to that of bacteriocins of the lactic acid bacteria and colicin V (Table 1.3). It was expected that MccE492 should be secreted by a dedicated ABC transporter system. However, genetic information for its export system has not been reported. The nucleotide sequence for the 24 amino acids at the N-terminus of *mccA* overlaps with the end of *mccB*, which encodes the 95-amino-acid immunity protein. Based on the comparison of the nucleotide sequence, Lagos *et al.* (1999) showed that MccE492 is homologous to microcin 24 with an identity of 52%. The immunity protein of MccE492 is similar in size and has an identity of 39% and a similarity of 56% to the Mcc24 immunity protein (Sable *et al.*, 2003). This suggests that both of these microcins belong to same family and probably have a common ancestry. No information is available about the export and mode of action of Mcc24.

Table 1. 3 Comparison of double-glycine-type leader peptides associated with colicin V, microcin H47 and microcin E492.

Colicin/Microcin	Sequence of leader peptide	Reference
Consensus	**LS--EL--I-GG X.**	Håvarstein *et al.*, 1994
Colicin V	MRT**LT**LN**EL**DS**V**S**GG A.**	Gilson *et al.*, 1990
Microcin H47	MRE**IT**ES**QL**RY**I**S**GA G.**	Rodriguez *et al.*, 1999
Microcin E492	MELRMRE**IS**QK**DL**NL**A**F**GA G.**	Lagos *et al.*, 1999

1.5 Applications for colicins

Researchers have isolated and characterized a diverse group of colicins from *E. coli*, but their potential for practical use is in question. The research has been narrowly focused on the biochemical, physiological and molecular aspects of colicins. Regardless of their exact role, it is obvious that colicins occur in relatively high frequencies in *E. coli* populations and that colicin-producing cells have competitive advantage over members of a microbial community by killing other organisms (Riley and Gordon, 1999). The most obvious advantage of colicins is their potential use to inhibit the growth of pathogenic strains of *E. coli* O157H7 and *Salmonella*. In contrast, research on bacteriocins from lactic acid bacteria has received enormous attention and their practical application as food biopreservatives has been studied in great detail. As a consequence of the intense investigation on bacteriocins of lactic acid bacteria, a large number of bacteriocins have been discovered and characterized, and the genetic mechanisms behind bacteriocin production have been determined. This has led to new preservation strategies for application in food systems.

Nisin, for example, is a bacteriocin from *L. lactis* subsp. *lactis* and has killing ability against a broad range of Gram-positive bacteria. Nisin is licensed for use as a food additive in over 54 countries (Delves-Broughton, 1990). Another bacteriocin of lactic acid bacteria, pediocin PA-1/AcH also has potential for use as a food preservative. It is protected by several U.S. and European patents, but is not in commercial use. In addition, two commercial compounds, Microgard and Alta 2341, which are fermentation products of food grade bacteria that impart antibacterial properties to foods, have been licensed for addition to foods. No other bacteriocins have been approved for addition to foods (Buard *et al.*, 2003; Lemay *et al.*, 2002; Stiles, 1996).

Numerous studies on application of pure bacteriocin, bacteriocin-producing

cultures or bacteriocin-containing fermentation products from a variety of lactic acid bacteria have been investigated for use in milk and dairy products, vegetable fermentations and meat products (Holzapfel *et al.*, 1995; McMullen and Stiles, 1996; Schillinger *et al.*, 1996). Our research group has also characterized several useful bacteriocins and examined the application of bacteriocinogenic lactic acid bacteria and their bacteriocins for the preservation of packaged meat products including ground beef (for detailed reviews, see McMullen and Stiles, 1996; Stiles, 1996).

During the last 20 years, the major focus of applied research on bacteriocins has been to inhibit the growth of foodborne pathogens, especially *Listeria monocytogenes* because of serious outbreaks of listeriosis that spurred a growing awareness of food safety concerns (Ennahar *et al.*, 1999). A large number of antilisterial bacteriocins have been identified and many of them have been well characterized. Most of these belong to class IIa bacteriocins, which are defined as small, heat resistant peptides that have a YGNGVXC sequence motif near the N-terminus. This group of bacteriocins appears to be the most promising candidates for application in the food industry, not only because of their strong antilisterial activity but also because of their relatively broad activity spectrum against other spoilage and foodborne pathogenic bacteria including spoilage lactic acid bacteria, *Brochothrix* spp. *Clostridium* spp. and *Bacillus* spp. (Eijsink *et al.*, 1998; Ennahar *et al.*, 1999). Unfortunately, the bacteriocins produced by lactic acid bacteria including nisin and pediocin PA-1/AcH are not active against Gram-negative bacteria, such as *E. coli* and *Salmonella* unless the cells are exposed to an additional sublethal stress (Cutter and Siragusa, 1995; Stevens *et al.*, 1991, 1992).

E. coli and *Salmonella* are of major concern in a wide variety of foods. Foodborne disease caused by these pathogens has caused considerable clinical and public health concern and significant economic loss. Among the five major groups of pathogenic *E. coli* that are currently associated with foodborne illness,

Enterohemorrhagic *E. coli* (EHEC) strains that include *E. coli* O157:H7 are an important pathogens. They cause serious illness in humans in the form of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). EHEC infections have most often been associated with outbreaks of foodborne illness from undercooked ground beef, contaminated vegetables and contaminated water. Other acidic foods such as mayonnaise, apple cider and yogurt have also been implicated in outbreaks of HC (Tsai and Ingham, 1997). *Salmonella* spp. can cause typhoid or enteric fever and severe gastroenteritis. Although animal-based foods are mostly involved in outbreaks of salmonellosis, apple cider has also been implicated (Leyer and Johnson, 1993; Tsai and Ingham, 1997). These Gram-negative foodborne pathogens are not sensitive to nisin or other classes of bacteriocins from lactic acid bacteria. In contrast, colicins, the classical bacteriocins of Gram-negative bacteria, differ from those of the lactic acid bacteria. They are specifically active against *E. coli* and other closely-related strains of *Enterobacteriaceae* (Pugsley and Oudega, 1987). The inhibitory activity of several colicins against *E. coli* O157:H7 and other pathogenic *E. coli* has been reported. Bradley *et al.* (1991) showed that colicins G, H, E2 and V strongly inhibit the growth of strains of EHEC. Murinda *et al.* (1996) showed similar results.

Several studies have shown that construction of a multiple bacteriocin producing system and heterologous expression of bacteriocins by various food grade lactic acid bacteria strains is possible (McCormick, 1998; Stiles, 1996; Van Belkum, 1996). This may offer an excellent tool to extend the application of bacteriocins in food preservation. Part of the research in our laboratory has focused on these strategies with special interest in the development of colicin-producing lactic acid bacteria. It is obvious that colicins that have strong activity against Gram-negative spoilage and pathogenic microorganisms have great potential for application in control of spoilage and pathogenic organisms in foods. It may be possible to apply well-characterized, non-toxic and purified colicins as

food preservatives. It may even be possible to design a useful tool incorporating bacteriocins from lactic acid bacteria with colicins from *E. coli* to control both undesirable Gram-positive and Gram-negative spoilage and pathogenic species using well-screened, food grade lactic acid bacteria as host strains.

1.6 Research objectives

The objective of this study was to obtain colicin-producing strains of *Enterobacteriaceae* from meats for which inhibitory activity of the growth of *E. coli* O157:H7 and *Salmonella* could be demonstrated. The overall goal of this study is to discover colicin(s) that can be used for heterologous expression in suitable lactic acid bacteria. Therefore, this study was conducted to achieve the following objectives:

1. Isolate and identify colicin-producing *Enterobacteriaceae* from meats
2. Characterize the newly identified colicin(s) with respect to activity spectrum, molecular size, chemical and physical stability, and conditions for production.
3. Purify a selected colicin and characterize the genetic determinants associated with its production, immunity and export.
4. Investigate the possibility of colicin production by heterologous expression system in lactic acid bacteria.

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CHAPTER 2

ISOLATION AND IDENTIFICATION OF COLICIN Y101 PRODUCED BY *Escherichia coli* ISOLATED FROM MEATS

2.1. Introduction

Bacteriocin production is a common phenomenon in bacteria playing a possible role of competitive interactions between microorganisms. Bacteriocins include colicins and microcins of *Enterobacteriaceae* as well as the lantibiotic and nonlantibiotic bacteriocins produced by Gram-positive bacteria (James *et al.*, 1992; Reeves, 1972). Colicins are the classical and best characterized bacteriocins. They are a heterologous group of proteins that are produced by *Escherichia coli* and closely related members of the family *Enterobacteriaceae* (Braun *et al.*, 1994; Luria and Suit, 1987). They have antimicrobial activity generally against closely related bacteria. Colicins produce "immunity" proteins to protect the producer organism against its homologous colicin but it does not mediate immunity against heterologous colicins. Typical characteristics of colicins include: (i) a narrow spectrum of antimicrobial activity; (ii) a bactericidal mode of action; (iii) receptor specificity required for its uptake; (iv) immunity protein specificity for host cell defense; (v) SOS inducibility of synthesis, except colicin V; (vi) plasmid-originated genetic determinant with the possible exception of colicin L; and (vii) large molecular size ranging from 29 kDa to 75 kDa with the exception of colicin V (Konisky, 1982; Lekey *et al.*, 1994; Pugsley, 1984a,b).

Colicin V has several distinguishing characters compared with other colicins. Its synthesis is not SOS inducible and its molecular size is much smaller than other colicins. Colicin V is a ribosomally synthesized antimicrobial peptide encoded by a large, low

copy number plasmid (Gilson *et al.*, 1990). Secretion of ColV requires dedicated export proteins instead of a lysis protein. ColV is equivalent to the class II bacteriocins of Gram-positive lactic acid bacteria (Klaenhammer, 1993; Nes *et al.*, 1996).

Little is known about the factors that determine the frequency of colicinogeny in natural populations. It was reported that over 30% of lactose-fermenting, Gram-negative isolates from the River Seine were colicin positive (Pugsley 1984a). It was also reported that 50% of the strains isolated from humans and 16% of animal isolates in the *E. coli* reference collection (ECOR) produced colicins (Riley and Gordon, 1996). Bradley (1991) indicated that 34% of serotype O111 strains evaluated were colicinogenic. This trait is more common among pathogenic than commensal isolates and may be more prevalent among human isolates than animal isolates (Riley and Gordon, 1996).

Controlling pathogenic organisms in food can reduce the risk of foodborne disease and enhance food safety. Foodborne disease causes significant economic loss each year. Attempts for the control of foodborne pathogens by bacteriocins in protective culture has been studied in several food systems (Holzapfel *et al.,* 1995; McMullen and Stiles. 1996; Schillinger *et al.,* 1996). The majority of methods for control were achieved with bacteriocinogenic lactic acid bacteria. Nisin produced by *Lactococcus lactis* has served as a model for the application of bacteriocins in food biopreservation. However, bacteriocins from Gram-positive bacteria including nisin are not active against Gram-negative bacteria without additional treatment to give a sublethal stress. Several studies demonstrated the successful expression of colicin V by lactic acid bacteria such as *L. lactis* and *Carnobacterium divergens* (McCormick *et al.*, 1999; Van Belkum *et al.*, 1996). Controlling pathogenic *E. coli* in vacuum-packaged chill-stored meat was investigated using *C. maltaromaticum* UAL26 producing colicin V suggesting as a prototype for the strategy of using LAB to control the growth of Gram-negative spoilage and pathogenic organisms (Watters, 2002). This indicates that it may not be possible to use colicins

produced by lactic acid bacteria in the control of pathogenic *E. coli* in meat.

The objective of this study was to isolate and identify colicins that could effectively inhibit Gram-negative spoilage or pathogenic bacteria for use in protective cultures. This chapter describes the isolation, identification and characterization of colicins produced by *E. coli* isolated from ground beef.

2.2. Materials and Methods

2.2.1. Media and maintenance of cultures

Nutrient broth (NB) and Luria-Bertani broth (LB) (Difco, BD Diagnostics, Sparks, MD) without addition of supplementary reagents were used in routine culture and maintenance of *E. coli* isolates from hamburger patties and commercially available ground beef in the Food Microbiology Laboratory, University of Alberta. All cultures other than *E. coli* isolates used in this study were grown in media and at temperatures indicated in Table 2.1. Soft and hard agars were prepared by adding 0.75% and 1.5% agar (wt./vol), respectively, to specified broth media. Stock cultures were kept in the media that were used to grow *E. coli* isolates with 15% glycerol (vol/vol) added and the cultures were stored at -80°C. Working cultures were maintained for no more than 2 weeks at 4°C on nutrient agar slants. When required, media were supplemented with increasing concentrations of mitomycin C (0.05, 0.1, 0.125 and 0.15 μg/mL; Sigma-Aldrich, Oakville, Ontario) to induce colicin production. Antibiotic supplements, if required, were added in the following concentrations: ampicillin 150 μg/mL (Sigma-Aldrich), chloramphenicol 30 μg/mL (Sigma-Aldrich), kanamycin 50 μg/mL (Sigma-Aldrich).

Table 2.1 Selected gram-negative bacterial strains used as indicators.

Stains[a]	Growth conditions[b]
Pseudomonas aeruginosa ATCC 14207	BHI, 30°C
Pseudomonas aeruginosa ATCC 15442	BHI, 30°C
Pseudomonas aeruginosa ATCC 27853	BHI, 30°C
Salmonella Choleraesuis ATCC 10708	TSB, 37°C
Salmonella Choleraesuis ATCC 13312	TSB, 37°C
Salmonella Enteritidis ATCC 13076	TSB, 37°C
Salmonella Gaminara ATCC 8324	TSB, 37°C
Salmonella Paratyphi ATCC 8759	TSB, 37°C
Salmonella Senftenberg ATCC 43845	TSB, 37°C
Salmonella Thompson ATCC 8391	TSB, 37°C
Salmonella Typhimurium ATCC 13311	TSB, 37°C
Salmonella Typhimurium ATCC 23564	TSB, 37°C
Salmonella Worthington ATCC 9607	TSB, 37°C
Yersinia enterocolitica ATCC 23715	TSB, 37°C
Shigella flexneri ATCC 12661	TSB, 37°C
Proteus vulgaris ATCC 13315	TSB, 37°C
Proteus vulgaris ATCC 8427	TSB, 37°C
Serratia marcescens ATCC 13880	LB, 30°C
Serratia marcescens NCIB 1377	LB, 30°C
Serratia liquefaciens ATCC 27592	LB, 30°C
Enterobacter agglomerans ATCC 27155	LB, 30°C
Enterobacter hafniae ATCC 13337	LB, 30°C
Enterobacter cloacae NCDO 612	LB, 30°C
Citrobacter freundii ATCC 8090	LB, 37°C
Citrobacter intermedium ATCC 6750	LB, 37°C
Klebsiella ozaenae ATCC 11296	LB, 37°C
Klebsiella pneumoniae ATCC 13883	LB, 37°C
Klebsiella rhinoscleromatis ATCC 13884	LB, 37°C
Escherichia coli O157:H7 12096*	LB, 37°C
Escherichia coli O157:H7 12902*	LB, 37°C
Escherichia coli O157:H7 13025*	LB, 37°C
Escherichia coli O157:H7 ATCC 43895	LB, 37°C
Escherichia coli ATCC 25922	LB, 37°C
Escherichia coli ATCC 11229	LB, 37°C

[a] ATCC, American Type Culture Collection, Manassas, VA, USA; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland, United Kingdom; NCDO, National Collection of Dairy Organisms, Aberdeen, Scotland, United Kingdom. *, supplied by Dr. J. Waters, Clinical Isolates, Provincial Laboratory of Public Health, Edmonton, Alberta.

[b] TSB, Tryticase Soy Broth (BBL, Becton Dickinson, Cockeysville, MD): BHI, Brain Heart Infusion broth (Difco, BD Diagnostics, Sparks, MD); LB, Luria-Bertani broth (Difco, BD Diagnostics, Sparks, MD).

2.2.2. Isolation of colicin-producing *E. coli* and activity assays

All *E. coli* isolates were screened for antibacterial activity by the deferred technique (Ahn and Stiles, 1990). Overnight cultures of *E. coli* were spotted onto triplicate plates of nutrient agar with a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario, Canada) and incubated at 37°C for 18 h. After growth, one plate was retained as a reference; the other plates were exposed to chloroform for 5 minutes to kill the "producer" strains. The chloroform was allowed to dissipate and the plates were overlayered with 7 mL of soft nutrient agar containing a 1% inoculum of *E. coli* DH5α (BRL Life Technologies, Rockville, MD) or *E. coli* MH1 (Casadaban and Cohen, 1980) as indicator strains for colicin production. These plates were incubated at 37°C for 18 h and examined for zones of inhibition. Individual colonies that showed inhibitory activity against either of the indicator strains were confirmed for antibacterial activity by the spot on lawn technique (Hastings *et al.*, 1991). A sample of supernatant from a freshly-grown culture of the producer strain was heated at 70°C for 30 min and serially diluted by two-fold dilutions. A 10 μL portion was spotted onto the surface of a freshly-prepared indicator lawn containing 1% inoculum of indicator cells in soft nutrient agar. Plates were incubated at 37°C for 18 h and zones of inhibition were measured. Positive samples were kept for further identification and characterization. Assays for colicin production and other tests for inhibitory activity were done in duplicate and were replicated 3 times. Inhibition was defined as the presence of a distinct, clear zone around the colony. The concentration of colicins was estimated by the critical dilution method (Schillinger *et al.*, 1993), and activity was expressed as arbitrary activity units per mL (AU/mL), where one arbitrary activity unit was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn, multiplied by hundred to obtain the AU/mL of the original sample. Pronase E (100 μg/mL, Sigma) was spotted

beside individual colonies or within the area of supernatant zone to confirm that the inhibitory substance was proteinaceous

2.2.3. Partial purification of colicins

The colicins produced by *E. coli* isolates were partially purified for further comparison and characterization. The producer strain was grown for 18 h at 37°C in 1 liter of either NB or LB. After growth, the culture was heated at 75°C for 30 min, followed by centrifugation at 11,700 x *g* for 40 min. One liter of heated culture supernatant was membrane-filtered (PVDF membrane, 0.2 μm pore size, Waters Limited, Mississauga, ON, Canada) and loaded onto an Amberlite XAD-8 (BDH Chemicals Ltd., Poole, UK) hydrophobic column (4 x 40 cm). The column was equilibrated with 1 liter of 0.1% trifluoroacetic acid (TFA) followed by elution with 0.5 liter of 20%, 60% and 80% ethanol solutions in 0.1% TFA at a flow rate of 5 mL/min. Active fractions from the XAD-8 column were pooled and concentrated by rotary evaporation, freeze dried, resuspended in 1.5 mL of 0.1% TFA and membrane-filtered (PVDF membrane, 0.22 μm pore size, Waters Limited, Mississauga, ON, Canada). This sample was applied to a 1 mL disposable hydrophobic silica SepPak C18 column (Waters Limited, Mississauga, ON, Canada) and eluted with an ethanol/water gradient. The active fraction was concentrated by Speed Vac concentrator, freeze-dried and resuspended in 0.5 mL of 0.1% TFA or sterile distilled water. This preparation was designated as "partially purified colicin" and it was used in further assays. Each of these preparations was assayed by the spot on lawn technique and the critical dilution method against designated *E. coli* indicator strains.

2.2.4. Assay of activity spectrum and immunity

For evaluation of the inhibitory activity the putative colicin producer strains were tested by deferred and spot-on-lawn inhibition tests (Ahn and Stiles, 1990) against *E. coli* isolates from hamburger patty and ground beef, and reference strains of *E. coli*, *Salmonella* and other *Enterobacteriaceae* listed in Table 2.1. The partially purified fractions were also tested against the same indicator strains. Colicin-producers were tested as both producer and indicator strains to establish whether the producer has immunity to itself and to assess its sensitivity to other producer strains.

2.2.5. Effect of heat, enzymes and pH on colicin activity

Culture supernatant and partially purified colicins were heated at 70°C for 30 min or at 85°C for 30 min in water baths and in boiling water for 5, 10, and 20 min and then cooled to room temperature. Colicin activity was determined immediately by spot on lawn test with *E. coli* DH5α as the indicator.

The following enzymes in appropriate buffer solutions were tested for their effect on colicin activity: α-chymotrypsin (pH 7.0; Serva, Heidelberg, Germany), trypsin (pH 7.0; Sigma-Aldrich, Oakville, Ontario), pepsin (pH 3.0, Merck, Darmstadt, Germany), proteinase K (pH 7.0; Sigma-Aldrich), α–amylase (pH 7.0; Sigma-Aldrich), lipase (pH 7.0; Sigma-Aldrich), catalase (pH 7.0; Sigma-Aldrich), and lysozyme (pH 7.0; Serva). Each enzyme solution was applied beside chloroform-inactivated colonies. The plates were allowed to stand for 30 min to evaporate the chloroform, the plates were overlayered with soft agar containing indicator *E. coli* DH5α and incubated for 18 h at 37°C. Partially purified supernatant and culture supernatant were also tested with enzymes. Each enzyme solution was added to the colicin samples and held for 2 h.

Enzymes were then inactivated by boiling for 5 min and solutions were assayed for colicin activity by the spot-on-lawn test.

To determine the sensitivity of colicin to pH, partially purified colicin and culture supernatant were adjusted to pH levels from pH 2 to 10 using 6 N sodium hydroxide or hydrochloric acid, and maintained under these conditions for 3 hours at room temperature. The colicin activity was then assayed by the critical dilution method against *E. coli* DH5α. To correct for inhibition due to pH, samples were treated with proteinase K before adjusting the pH and tested against the same indicator strains. For each test, untreated samples were used and assayed as controls.

2.2.6. Molecular weight determination

Approximate molecular weights of partially purified colicins were determined on 20% polyacrylamide gel with the buffer system described by Laemmli (1970). After samples were loaded in duplicate and the reference proteins were loaded onto the gel, electrophoresis was done at 150 volts. Gels were washed and fixed in 50% methanol (vol/vol) for 1 h and either stained with Coomassie Brilliant Blue (Bio-Rad Laboratories Ltd., Mississauga. ON) or assayed for colicin activity by the method of Barefoot and Klaenhammer (1984). To assay for colicin activity, the gel was placed onto a nutrient agar plate and overlayered with soft agar containing 1% inoculum of indicator *E. coli* DH5α. The plate was incubated at 37°C overnight and examined for zones of inhibition.

In some cases, mini-Tricin-SDS-PAGE gels were used following the method of Schägger and von Jagow (1987). Protein standards used and their individual molecular weights are as follows: Myosin, 200,000; β-galactosidase, 116,250; Phosphorylase b, 97,400; Serum albumin 66,200; Ovalbumin, 45,000; Carbonic anhydrase, 31,000; Trypsin inhibitor 21,500; Lysozyme, 14,000; Aprotinin, 6,500 (all obtained from Bio-Rad

Laboratories Ltd., Mississauga, ON).

2.2.7. Plasmid isolation and electrotransformation

Colicinogenic *E. coli* isolates were grown in LB to log phase and harvested by centrifugation (8,000 x *g* for 15 min). Plasmid DNA was isolated and separated by horizontal 0.7% agarose gel electrophoresis using TAE buffer as described by Sambrook *et al.* (1989). The size of the plasmid was estimated by comparison to the eight plasmid species extracted from *E. coli* V517 as described by Macrina *et al.* (1978).

Electrotransformation was done with the Bio-Rad gene pulser (25 μF and 250 kV) and Bio-Rad pulse controller (200 ohms) (Bio-Rad Laboratories Ltd., Mississauga, ON). Competent cells of *E. coli* DH5α and MH1 were prepared and transformed according to the one-step method of Chung *et al.* (1989). The transformed cells were plated on LB plates containing partially purified colicin of *E. coli* KB101 and incubated at 37°C overnight. Survivors were screened for the production of colicin against indicator strains by the deferred inhibition test.

2.3. Results

2.3.1. Isolation of colicin-producing *E. coli* and their inhibitory activity

A total of 264 and 114 colonies of *E. coli* was isolated from ground beef and hamburger patties, respectively, with the help of Jonathan Lai and Julie Prokuda in Food Microbiology laboratory. All isolates were screened for colicin production against *E. coli* DH5α and MH1 with and without addition of mitomycin C. The data in Table 2.2 show the number of colicin-positive isolates detected on different growth media. The Colicin V

producer *E. coli* MH1 carrying plasmid pHK22 (Fath *et al.,* 1992; Gilson *et al.*, 1987) was used as a positive control. Approximately 20% of the 378 isolates produced clear zones of inhibition without mitomycin C induction (Table 2.2). When mitomycin C was added to the agar plates to induce colicin production, the number of positive isolates did not change but larger zones of inhibition were observed. Best results for colicin production were observed on Nutrient agar.

Table 2.2 Frequency of detection of colicin-positive strains[a].

Source	Number of strains tested	Number of strains that demonstrated activity[b]:		
		NB	TSB	LB
Ground beef	264	58	52	53
Hamburger patties	114	19	17	17
Total	**378**	**77**	**69**	**70**

[a] *E. coli* DH5α and MH1 were used as indicators. Only the number of strains that showed a zone of inhibition against both indicator strains were counted as positive isolates.

[b] NB, Nutrient Broth (Difco, BD Diagnostics, Sparks, MD); TSB, Trypticase Soy Broth (BBL, Becton Dickinson, Cockeysville, MD); LB, Luria-Bertani broth (Difco, BD Diagnostics, Sparks, MD).

All of the presumptive colicin producers were tested against a wide range of strains of *E. coli* including 30 randomly selected wild-type (WT) *E. coli* isolated from meat, 2 ATCC strains, and 8 strains from our culture collection. From these producer

strains a total of eight strains that exhibited the broadest inhibitory spectrum was selected (Data not shown). Further study of their activity spectra was tested against the 378 WT *E. coli* isolates from meat sources, 94 *Salmonella* species isolated from animal and human sources (donated by Dr. M. Finlayson, Department of Medical Microbiology, University of Alberta), 3 *Pseudomonas* strains, 24 ATCC strains of *Enterobacteriaceae*, and 4 ATCC strains of *E. coli* O157:H7. The data in Table 2.3 show the number of *E. coli* isolates and *Salmonella* species that were sensitive to these eight producer strains. The activity profile of these producer strains against the bacteria listed in Table 2.4 showed that *E. coli* KH35, KB81 and KB101 had relatively large activity spectra.

Table 2.3 Activity of *E. coli* isolates against *E. coli* and *Salmonella* determined by deferred inhibition test.

Selected producer strains	Number of strains inhibited	
	E. coli[a]	*Salmonella*[b]
E. coli KH15	75	26
E. coli KH21	132	29
E. coli KH35	241	56
E. coli KH65	86	25
E. coli KH72	79	36
E. coli KB31	105	31
E. coli KB81	223	28
E. coli KB101	226	58

[a]: 378 *E. coli* isolated from meat sources were tested.
[b]: 94 strains were tested, which were obtained from Dr. M. Finlayson.

Table 2.4 The inhibitory spectrum of colicin-producers determined by deferred inhibition test.

Indicators	Colicin positive producers of *E. coli**							
	KH15	KH21	KH35	KH65	KH72	KB31	KB81	KB101
Pseudomonas aeruginosa ATCC 15442								++
Pseudomonas aeruginosa ATCC 27853		+	+	+	+	+	+	++
Salmonella Choleraesuis ATCC 10708			+					+++
Salmonella Choleraesuis ATCC 13312			+					+++
Salmonella Enteritidis ATCC 13076			+	++			+	+++
Salmonella Gaminara ATCC 8324			+					+++
Salmonella Paratyphi ATCC 8759				++				
Salmonella Thompson ATCC 8391			+					++
Salmonella Typhimurium ATCC 13311			+				+	++
Salmonella Worthington ATCC 9607			+				+	++
Yersinia enterocolitica ATCC 23715				+		++	+	
Shigella flexneri ATCC 12661	++	++	+++	+++	+	+	+	++
Proteus vulgaris ATCC 8427				+		+		
Enterobacter agglomerans ATCC 27155				+		++		
Citrobacter freundii ATCC 8090	++	++	++	++	+	++	+	
Citrobacter intermedium ATCC 6750	++	++	++	+++	++	+	++	+
Klebsiella ozaenae ATCC 11296			+			++	+	+
Escherichia coli O157:H7 12096			++	++		++	+++	+++
Escherichia coli O157:H7 12902			++	++		++	+++	+++
Escherichia coli O157:H7 13025			++	++		++	+++	+++
Escherichia coli O157:H7 ATCC 43895			++	++		++	+++	+++

*: All colicin-positive strains were tested against general indicators of *E. coli* DH5α and MH1 and they showed large, clear zones of inhibition. ColV was used as a positive control. Zones of inhibition were expressed as: +, 5-10 mm; ++, 11-15 mm; +++, >15 mm.

2.3.2. Interrelatedness of selected producer strains

Colicin-producing strains are protected from their own colicins by a highly specific, co-transcribed immunity protein. The immunity protein for one type of colicin does not confer immunity to other types of colicin. More than 25 different colicins have been distinguished by the absence of cross-immunity between the producing strains. On this basis, eight colicin-positive producer strains were selected and examined for immunity to the colicins that they produce. Each producer strain was immune to its own colicins and they did not show cross-immunity to the other colicins (Table 2.5). In addition, their activity spectrum against test organisms was different (data not shown). Therefore, it is clear that the antimicrobial compounds produced by these strains are distinct.

Table 2.5 Cross-reactions between activity patterns of eight selected colicin-producing strains of *E. coli* determined by the deferred inhibition test.

***E. coli* indicator Strains**	***E coli* producer strains**							
	KH15	**KH21**	**KH35**	**KH65**	**KH72**	**KB31**	**KB81**	**KB101**
KH15	-	-	+	+	-	+	+	+
KH21	-	-	+	+	-	+	+	+
KH35	-	+	-	+	+	-	+	+
KH65	+	+	+	-	+	-	+	+
KH72	-	-	+	+	-	+	+	+
KB31	+	+	+	+	+	-	+	+
KB81	-	-	-	-	-	-	-	-
KB101	-	-	+	-	-	-	-	-

2.3.3. Molecular weight determination

The molecular weight of each of the colicins was estimated by SDS-PAGE gel electrophoresis. The colicin produced by each strain was partially purified from the culture supernatant. After electrophoresis, gels were stained with Coomassie Brilliant Blue or assayed for antimicrobial activity by overlayering the gel with an indicator lawn. Antimicrobial compounds on the gel developed a clear zone of inhibition against indicator *E. coli* DH5α. The zone of inhibition was matched against the stained band on the other gel and the molecular weight was estimated from protein markers (Bio-Rad Laboratories Ltd., Mississauga, ON) of known size (Data not shown). The estimated molecular size of the eight antimicrobial compounds is listed in Table 2.6.

Antimicrobial agents produced by isolates KH15, KH35, KH65, KH72 and KB31 were approximately 66 kDa, which corresponds with the molecular size of the colicin E group (Pugsley 1984a). The molecular size of the antimicrobial agents produced by strains KH21 and KB81 was about 29 kDa (Fig. 2.1). The only known colicin with 29 kDa is colicin M (Harkness and Ölschläger, 1991; Köck *et al.*, 1987). Unlike the others, the molecular size of antimicrobial substance produced by KB101 was about 8 kDa (Fig. 2.1). The unusually small molecular size of this substance made it equivalent to the smallest known colicin, colicin V that is 8.7 kDa (Fath *et al.*, 1994).

Table 2.6 Estimated molecular size (kDa) of the putative colicins.

E. coli:	KH15	KH21	KH35	KH65	KH72	KB31	KB81	KB101
Approx. Molecular Size (kDa)	66	29	66	66	66	66	29	8

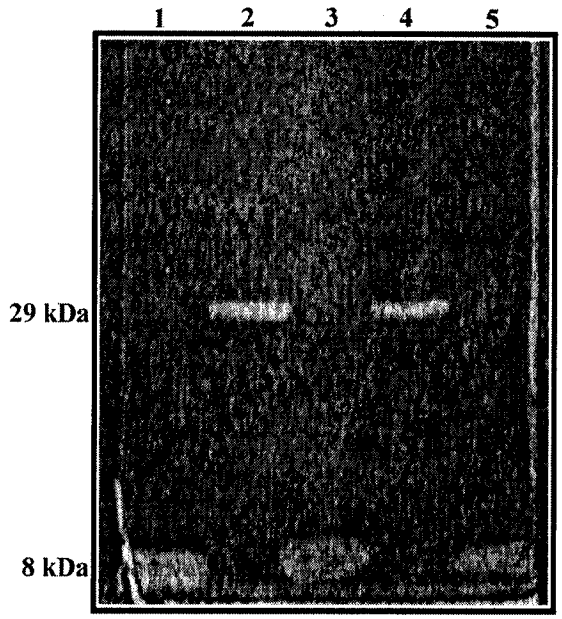

Figure 2.1 Localization of inhibitory activity against *E. coli* DH5α by colicins produced by *E. coli* KH21 (lane 2), KB81 (lane 4) and KB101 (lanes 1, 3 and 5).

2.3.4. Preliminary characterization of colicin produced by *E. coli* KB101

From the preliminary results of deferred and spot-on-lawn inhibitory tests and SDS-PAGE electrophoresis, it was noted that the antibacterial substance produced by *E. coli* KB101 had a relatively broad antimicrobial activity against *Enterobacteriaceae* including *E. coli* O157:H7 and *Salmonella* species (Tables 2. 3 and 2. 4) and had a small molecular size. Hence, the inhibitory substance produced by *E. coli* KB101 was chosen for further study and tentatively designated as colicin Y101 (ColY101). Amongst colicins, the molecular size of colicin V (ColV) and colicin G (ColG) are known to be 8.7 kDa and 5.5 kDa, respectively. Production of ColV and G are not induced by mitomycin C (Bradley, 1991; Fath *et al*, 1994). Because of similar molecular weight to ColV and ColG and no induction by mitomycin C, immunity of ColY101 to ColV and ColG was

examined. The ColG producer strain was not sensitive to, but it was active against, *E. coli* KB101 (data not shown). Growth of *E. coli* KB101 was not inhibited by ColV; however, a weak zone of inhibition was observed when *E. coli* KB101 was overlayered with soft agar containing the ColV producer strain. It was clear that ColV might be immune to ColY101 but not fully protected (data not shown). Therefore, it was concluded that ColY101 was not the same as ColG but that it might be similar but not necessarily identical to ColV. Chemical and genetic evidence would be required to prove this.

Antagonistic activity of partially purified ColY101 against indicator strain *E. coli* DH5α and MH1 was inactivated by treatment with proteolytic enzymes (Table 2.7), including α-chymotrypsin, trypsin, pepsin and proteinase K. Proteolytic enzymes applied beside colonies on the deferred test inactivated colicin Y101 (Fig. 2.2). Treatment with catalase, lysozyme, α-amylase and lipase did not inactivate ColY101 (Table 2.7). Thermal stability of ColY101 was determined by heating partially purified ColY101 at different temperatures and measuring the residual activity by the critical dilution method. Values were determined by triplicate tests. The activity of ColY101 remained the same after heat treatment at 70°C for 30 min and 85°C for 30 min, and after immersion in a boiling water bath for 10 min. However, activity was decreased from 25,600 to 12,800 AU/mL after 20 min in a boiling water bath. Partially purified ColY101 was most active between pH 4.0 and 9.0. Activity was slightly decreased at pH levels above 9.0 and below 4.0, but considerable activity (6,400 AU/ml) could still be detected (Table 2.7).

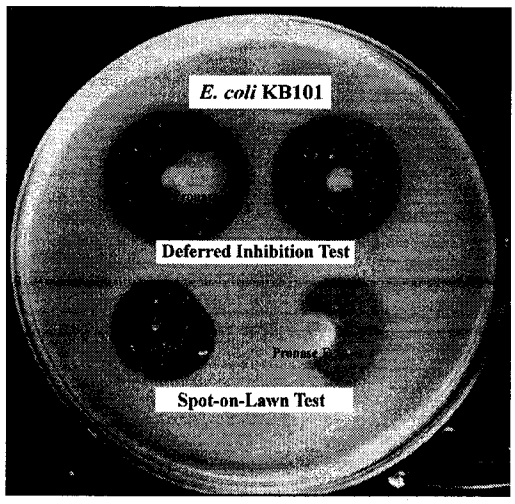

Figure 2. 2 Deferred inhibition and spot-on-lawn assay of *E. coli* KB101 against *E. coli* DH5α.

Table 2.7 Effect of selected enzymes, heat and pH treatment on inhibitory activity of ColY101 detected by doubling dilutions.

Treatment	Arbitrary Activity (AU/mL)
Control (untreated)	25,600
Enzymes	
α-chymotrypsin	0
Trypsin	0
Pepsin	0
Proteinase K	0
α-amylase	25,600
Lysozyme	25,600
Catalase	25,600
Lipase	25,600
Heat treatment	
70°C for 30 min	25,600
85°C for 30 min	25,600
Boiling for 10 min	25,600
Boiling for 20 min	12,800
pH	
2 - 3	6,400
4 - 9	25,600
10 - 11	6,400

2.3.5. Plasmid origin of ColY101

Colicins are typically encoded on plasmids with the possible exception of colicin L that appears to be chromosomally encoded (Hughes *et al.*, 1978). To determine the plasmid origin of ColY101, plasmid DNA was extracted from *E coli* KB101 and used to electrotransform competent strains of *E. coli* DH5α. Agarose gel electrophoresis of plasmid DNA isolated from *E. coli* KB101 revealed 3 plasmid bands, pKY1, pKY2, and pKY3, from largest to smallest.

A mixture of the three plasmids was electrotransformed into *E. coli* DH5α. A total of 15 colonies were obtained on plates containing ColY101 and they were examined for colicin activity against non-transformed *E. coli* DH5α. All of the transformants were colicin-positive and they were not sensitive to *E. coli* KB101. Plasmid isolation from a representative clone showed that ColY101-positive strains contained pKY1. Many tests were done to isolate transformants that contained pKY2 and pKY3. Three representative isolates containing pKY1, pKY2, and pKY3, separately, were examined for colicin activity and immunity (Table 2.8). *E. coli* DH5α carrying pKY1 was ColY101 positive and had immunity to ColY101. The isolates containing pKY2 or pKY3 were not active against *E. coli* DH5α and they were sensitive to ColY101 (Fig 2.2). Therefore, it was clear that ColY101 is plasmid-encoded.

Table 2.8 Inhibitory activity of transformants containing plasmid DNA isolated from *E. coli* KB101.

Producer	Indicator *E. coli*:				
	DH5α	KB101	KY1 (pKY1)	KY2 (pKY2)	KY3 (pKY3)
KB101	+	-	-	+	+
KY1 (pKY1)	+	-	-	+	+
KY2 (pKY2)	-	-	-	-	-
KY3 (pKY3)	-	-	-	-	-

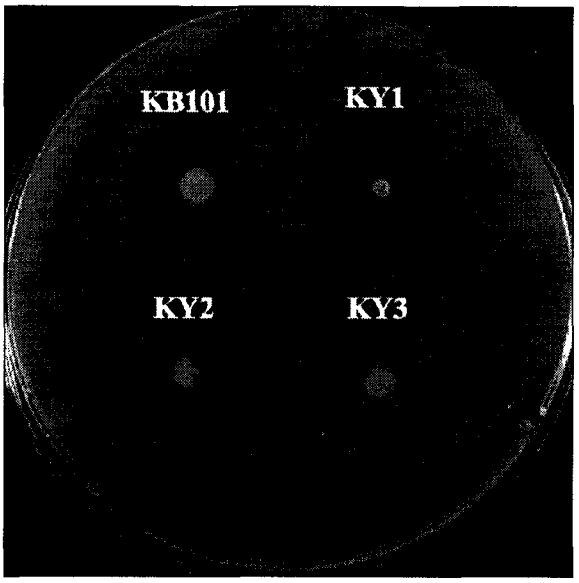

Figure 2.3 Deferred inhibition test with *E. coli* KB101 and *E. coli* DH5α containing pKY1, pKY2, and pKY3 against *E. coli* DH5α as the indicator strains.

2. 4. Discussion

Several studies of colicin evolution and ecology revealed that colicinogeny in *E. coli* populations is more common among pathogenic than commensal isolates and seemed to be more prevalent among human than animal isolates (Riley and Gordon, 1992, 1996). Although the reported frequency of colicinogenic isolates varies widely, the frequency depends on different sample types, growth media and indicator strains used. (Pugsley, 1984a; Riley and Gordon, 1992). Little is known about the frequency of colicinogeny in natural populations. It is generally accepted that over 25% of natural and clinical isolates of *E. coli* are colicinogenic (Hardy, 1974; Murinda *et al.*, 1996; Pugsley, 1984a). The result of this study indicated that 20% of *E. coli* isolates from meat were colicinogenic. One of the peculiarities of colicin production, with the exception of

colicins V and G, is that synthesis is induced by the so-called 'SOS' response. 'SOS' induciblility may be a factor in production of most colicins (Bradley, 1991; Gilson *et al.*, 1990; Luria and Suit, 1987). The growth medium and glucose concentration may be key factors affecting the amount of colicin produced (Nakazawa and Tamada, 1972; Spangler *et al.*, 1985). The three different media NB, LB and TSB with or without mitomycin C induction in this study did not make a noticeable difference to the number of positive isolates. However, the size of the zone of inhibition was greater when mitomycin C was added to the culture. The inhibitory agent produced by KB101 was not affected by mitomycin C indicating that strain KB101 was not SOS inducible. Nutrient broth was selected for use in most experiments because colicin production in this medium was the best among the three media.

Colicins generally have very narrow activity spectrum against *E. coli* and closely related bacteria. Their activity is limited to sensitive strains within the species of the producer organism depending upon receptor specificity and immunity (Braun *et al.*, 1994; Konisky, 1982). The specific immunity protein of each colicin is a key factor to identify new and/or different types of colicins. Colicin-producing bacteria are protected from their own colicin action both endogenously and exogenously because of cotranscription of a highly specific immunity protein. However, the immunity protein to the same type of colicin does not confer immunity to other types of colicin. This is referred to as cross-immunity and more than 25 different colicins have been distinguished by the absence of cross-immunity between the producing strains (Davies and Reeves, 1975a,b; Lazdunski, 1998; Pugsley, 1985).

Of the eight isolates selected in this study, the four weak producers were eliminated (Tables 2.3 and 2.4). Although KH35, KH65, and KB81 effectively inhibited the growth of the indicators, these three producers were eliminated because of the large molecular size of their inhibitory compounds. The antimicrobial compound of KB101

was of special interest because of its atypically small size and its activity against a large number of *Enterobacteriaceae,* including *E. coli* O157:H7, *Salmonella, Shigella*, and *Klebsiella*. It was also active against *P. aeruginosa, S. flexneri, K. ozaenae* and *C. intermedium.* The molecular size of the colicin produced by KB101 was about 8 kDa measured by SDS-PAGE. Colicin V is known as the smallest colicin with a molecular weight of 8735.8 Da (Fath *et al.,* 1994) and its synthesis is not 'SOS' inducible. Although the molecular size of colicin G was suggested to be smaller (5.5 kDa) (Bradley, 1991), no structural or genetic evidence has been reported for this to date. Colicin G production was also not affected by 'SOS' response. Because of the similar molecular size and the lack of induction by mitomycin C, the cross-immunity between ColY101, ColV and ColG was also studied. ColY101 was not active against either the ColV or the ColG producers. *E. coli* KB101 was sensitive to ColG but not to ColV. Therefore, it is clear that ColY101 is not identical to ColG.

ColY101 is proteinaceous and insensitive to lipase, catalase and α-amylase. It is stable at temperatures above 70°C up to boiling, but it was not stable at extremes of pH (pH2 and pH10). In addition to heat stability and broad activity spectrum, ColY101 stability in a wide pH range could be a potential advantage in its application in food. Preliminary data described in this chapter suggest that colicins in combination with other components may provide an effective method for controlling *E. coli* O157:H7 and other spoilage organisms in food preservation and processing.

Colicins are plasmid-determined antibacterial proteins with the possible exception being that colicin L is chromosomally encoded (Pugsley & Oudega, 1987). ColY101 was shown to be plasmid encoded. Strain KB101 has three plasmids, pKY1, pKY2, and pKY3, all of which were transferred to *E. coli* DH5α and MH1. Transformants containing pKY1 were ColY101-positive and immune to *E. coli* KB101, whereas two other transformants with pKY2 or pKY3 were negative and they were

sensitive to *E. coli* KB101. The transformant carrying pKY1 was examined for its activity spectrum and it was shown to have the same antibacterial profile as *E. coli* KB101. Therefore, it is clear that colY101 is encoded on pKY1 that can be used for further genetic characterization.

Unlike most colicins, ColV does not utilize a lysis protein for its release. ColV encodes a set of dedicated export proteins required for its secretion (Fath & Kolter 1993; Gilson *et al.*, 1990). ColV is post-translationally modified protein by cleavage of its leader peptide and its leader peptide is similar to those of lactococcins and other class II bacteriocins of lactic acid bacteria. The prebacteriocins are cleaved immediately following a Gly-Gly couplet (Håvarstein *et al*, 1994). With the similar characteristics of colicin V to class II bacteriocins of lactic acid bacteria, the expression of colicin V genes in *L. lactis*, the expression of ColV in Gram-positive bacteria has been achieved (McCormick, 1999; Van Belkum *et al.*, 1996). These results indicate the possibility of practical application for controlling pathogenic organisms in food systems and animal diseases incorporating bacteriocins from both gram-positive and gram-negative bacteria (Stevens *et al.*, 1991, 1992). It is noteworthy that colicins with a broader spectrum of activity, which can inhibit organisms of practical significance (e.g., *E. coli* O157:H7 and *Salmonella*) can be transferred to food-grade lactic acid bacteria such as *Lactococcus* and *Carnobacterium,* which can be added to foods. Therefore, additional genetic characterization and cloning of colicin genes into food-grade plasmids is required for safe utilization in food systems. For this reason, purification and amino acid sequence of ColY101 was undertaken. Results from purification and cloning experiments are presented in Chapter 3.

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CHAPTER 3

PURIFICATION, IDENTIFICATION AND GENETIC CHARACTERIZATION OF COLICIN Y101

3.1. Introduction

Colicin V (ColV) produced by *Escherichia coli* displays several peculiar properties compared with other colicins. Its synthesis is not SOS inducible, it does not use a lysis protein for its release and it is considerably smaller than other colicins. It has a molecular weight of 8.7 kDa (Fath *et al.,* 1994; Gilson, *et al.,* 1990) while other colicins range in molecular size from 29 to 75 kDa. Most colicins are released from the cell by a specific lysis protein that causes cells to break or leak (Braun *et al.*, 1994; Lazdunski *et al.*, 1998), whereas ColV has a specific transport system for its secretion. It was therefore concluded that ColV might belong to the family of antimicrobial peptides known as microcins but it retained its original name for historical reasons (Fath *et al.,* 1992; Kolter and Moreno, 1992). However, the characteristics of ColV do not fit most of the general criteria for microcins and it has more in common with the non-lantibiotic class II bacteriocins produced by lactic acid bacteria (Håvarstein *et al.,* 1995; Nes *et al.,* 1996).

As with colicins, microcins are mostly plasmid encoded with the exception of microcins H47 and E492 (Baquero and Moreno, 1984; Lagos *et al.*, 1999 Lavina *et al.*, 1990). Unlike most colicins, microcins are defined as low-molecular-weight antimicrobial peptides less than 6 kDa. Their synthesis is non-lethal for the producing cells and it is not mediated by "SOS" repair pathway. Most microcins are synthesized as peptide precursors with a N-terminal leader sequence. The microcin precursors

subsequently undergo post-translational modification to generate active moieties before being exported from the cell (Asensio *et al.*, 1976; Baquero *et al.*, 1978; Kolter and Moreno, 1992). The role of leader peptides of microcins does not appear to be the same as in ColV and other class II bacteriocins of lactic acid bacteria. It does not appear to be involved in export of the microcin from the cell nor does it share significant homology with consensus sequence found in the double glycine leader peptides. The leader peptides of microcins are removed during peptide maturation (Garrido *et al.,* 1988; Madison *et al.,* 1997; Yorgey *et al.,* 1993).

ColV is encoded on large, low-copy-number plasmids. ColV plasmids display considerable diversity and range in size from 80 to 180 kb. They have been found in many strains of *E. coli*. Four linked genes from plasmid pColV-K30 are required for synthesis, immunity and export of ColV from the cell. Two genes encode ColV structural peptide (*cvaC*) and immunity (*cvi*). They are in a single operon while the two genes for the export proteins, *cvaA* and *cvaB*, are encoded in an operon that is oriented in the opposite direction. The ColV immunity protein is encoded by *cvi* that is located immediately upstream of the *cvaC* structural gene (Gilson *et al.,* 1990).

ColV consists of a 103-amino-acid pre-peptide (pre-ColV). It does not contain a typical *sec*-dependent N-terminal signal peptide. Instead, the ColV precursor contains a 15-amino-acid N-terminal extension with a Gly-Gly cleavage site. This has significant similarity to the double-glycine-type leader peptides of class II bacteriocins of lactic acid bacteria (Håvarstein *et al.*, 1994). The leader peptide of ColV and non-lantibiotic bacteriocins such as pediocin PA-1/AcH, leucocin A, lactococcin A and sakacin A provide the recognition signal for the dedicated transport system that involves two membrane-bound proteins (Stoddard *et al.,* 1992; Van Belkum *et al.*, 1997). The ColV leader peptide is removed concomitant with export of the colicin from the cell, yielding the 88-amino-acid mature peptide of ColV. Secretion of ColV is mediated by two dedicated export proteins, CvaA and CvaB, encoded by *cvaAB* operon with the support

of the outer membrane protein TolC that is encoded by a chromosomal gene (Fath and Kolter, 1993). CvaA is a member of the MFP family of proteins and is related to HlyD, while CvaB is a member of the ATP-binding cassette (ABC) exporter proteins that has a C-terminal ATP-binding domain and is known to be related to HlyB. Both HlyB and HlyD are responsible for the secretion of the α-hemolysin from *E. coli* (Holland *et al.*, 1990; Hughes *et al.*, 1992).

The work reported in this chapter was done with the purpose of finding a low molecular weight, heat stable colicin that could effectively inhibit *E. coli* O157:H7, *Salmonella* and other *Enterobacteriaceae*. We isolated *E. coli* KB101 with antibacterial activity from ground beef and tentatively named it colicin Y101. It has similarities with colicin V. Colicin Y101 is a non-SOS inducible, proteinaceous inhibitory substance. It is heat stable and has a molecular size about 8 kDa. Preliminary characteristics of colicin Y101 are described in chapter 2. In this chapter, the purification and the complete genetic analysis of colicin Y101 is reported.

3.2. Materials and Methods

3.2.1. Bacterial strains and culture conditions

E. coli strains and plasmids used in this study are listed in Table 3.1. The strains of *E. coli* used in this study, unless otherwise indicated, were DH5α (BRL Life Technologies, Rockville, MD) and MH1 (Casadaban and Cohen, 1980). They were grown in nutrient broth (NB) and Luria-Bertani broth (LB) (Difco, BD Diagnostics, Sparks, MD) on a rotary shaker-incubator at 250 rpm at 37°C. Working cultures were maintained for less than 2 weeks on NB or LB agar plates (1.5% added agar, Difco) at 4°C. Working cultures were subcultured in NB or LB at 37°C on two consecutive days before use in an experiment. Stock cultures were made in the same medium used to grow

Table 3.1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains:		
E. coli DH5α	F-Φ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1*hsd*R17 ($r_k^-m_k^+$) *pho*A *sup*E44 - *thi*-1*gyr*A96 *rel*A1, colicin sensitive	BRL Life Technology Inc.
E. coli MH1	MC1061 derivative; *ara*D139 *lac*X74 *gal*U *gal*K *hsr hsm*$^+$ *str*A colicin sensitive	Casadaban and Cohen (1980)
E. coli KB101	Wild type ColY101$^+$, contains pKY1, pKY2, pKY3	This study
E. coli KY1	*E. coli* DH5α-containing pKY1, ColY101$^+$	Chapter 2
Plasmids:		
pUC118	*lacZ'*, Amp^r, 3.2 kb	Vieira and Messing (1987)
pMG36e	Expression vector, Em^r, 3.6 kb	Van de Guchte *et al.* (1989)
pHK22	pACYC184 derivative containing 9.4 kb ColV gene cluster, *cvaA, cvaB, cvaC, cvi*, Cm^r, 13.6 kb	Gilson *et al* 1987
pKY1	ColY101$^+$, 120 kb	This study
pKY5	pMG36e containing 22.9 kb *Sac*I fragment from pKY1; ColY101$^+$, Em^r	This study
pKY7	pMG36e containing 14.6 kb *Sac*I fragment from pKY1; ColY101$^+$, Em^r	This study
pKY9	pUC118 containing 6.6 kb *Hin*dIII fragment from pKY1; ColY101$^+$, Amp^r	This study
pKY10	pUC118 containing 18.1 kb *Hin*dIII-*Sac*I fragment from pKY5; ColY101$^-$, Imm$^-$, Amp^r	This study
pKY11	pUC118 containing 8.3 kb *Hin*dIII-*Sac*I fragment from pKY5; ColY101$^-$, Imm$^-$, Amp^r	This study
pKY12	pUC118 containing 12.6 kb *Sac*I-*Hin*dIII fragment from pKY5; ColY101$^+$, Imm$^+$, Amp^r	This study
pKY20	pUC118 containing 2.0 kb *Hin*dIII-*Eco*RI fragment from pKY9; *cya, cyi*, Amp^r	This study
pKY29	pUC118 containing 2.9 kb *Hin*dIII-*Sac*I fragment from pKY9; *cya, cyi*, Amp^r	This study

pKY37	pUC118 containing 3.7 kb *Sac*I-*Hin*dIII fragment from pKY9; ColY101 sensitive, Amp^r	This study
pKY45	pKY9 with Δ(*Sac*I-*Sac*I), cya, cyi, Amp^r	This study
pKY50	pUC118 containing 5.0 kb *Hin*dIII-*Sac*I fragment from pKY9; $ColY101^+$, Amp^r	This study

[a] *cvaA/B*; colicin V export gene; *cvaC*, colicin V structural gene; *cvi*, colicin V immunity gene; *cya*, colicin Y101 structural gene; *cyi*, colicin Y101 immunity gene; Δ(*Sac*I-*Sac*I), deleted fragment between *Sac*I-*Sac*I restriction site; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.

cells by adding 15% (vol/vol) glycerol and stored at -80°C.

Antibiotics were added as selective agents where required: ampicillin 150 μg/ml (Sigma-Aldrich, Oakville, Ontario), chloramphenicol 30 μg/ml (Sigma-Aldrich), kanamycin 50 μg/ml (Sigma-Aldrich) and erythromycin 200 μg/ml (Sigma-Aldrich).

3.2.2. Colicin activity assay

Colicin activity of both parental and mutant strains of *E. coli* was monitored by deferred inhibition test as described previously (Chapter 2). After incubation of designated producer strains at 37°C for 18 h, indicator strains were inoculated (1%) from a fresh overnight culture into appropriate soft (0.75%) agar medium, overlayered onto the chloroform-inactivated producer strain(s) and plates were incubated at 37°C overnight. Quantification of colicin activity from culture supernatant and samples obtained from purification was determined by the critical dilution method (Chapter 2). Unless otherwise stated, *E. coli* DH5α and MH1 were used as the indicator strains for colicin activity assays. To confirm that the inhibitory substance is proteinaceous, pronase E was spotted beside the producer colony or adjacent to the area of spotted supernatant.

3.2.3. Purification of colicin Y101

The producer strain *E. coli* KB101 was grown in 4 liters of NB on a rotary shaker at 250 rpm at 37°C. After 18 h, the culture was heated at 80°C for 30 min, cooled to room temperature and the culture supernatant was collected by centrifugation at 11,700 x *g* for 40 min (Fraction I).

a) **Hydrophobic chromatography.** Fraction I (4 liters) was loaded directly onto an Amberlite XAD-8 column (150 x 75 mm: BDH Chemicals Ltd., Poole, UK) and washed with 3.5 liters of 0.1% trifluoroacetic acid (TFA), followed by 2 liters of 20% and 40% ethanol in 0.1% TFA (flow rate: 5 ml/min). The colicin active fraction was eluted with successive 2 liters of 60% ethanol in 0.1% TFA (flow rate: 5 ml/min) and concentrated to 320 ml by rotary evaporation (Fraction II).

b) **Cation-exchange chromatography.** Fraction II was applied to a SP Sepharose® fast flow cation exchange column (110 x 15 mm: Pharmacia Biotech, Baie d'Urfe, PQ) pre-equilibrated with 20 mM sodium acetate buffer at pH 5.0. The column was washed with 100 ml of sodium acetate buffer, followed by 50 ml of buffer containing different concentrations (50, 100, 500 mM, and 1 and 1.5 M) of sodium chloride. Most colicin was eluted with buffer containing 500 mM NaCl. The active eluant was desalted on a Sep-Pak C18 reverse-phase column (Waters Ltd., Mississauga, ON) according to the manufacturer's instructions. The column was washed with 20 ml of distilled water and the active compound was eluted with 10 ml of 20, 40, 60 and 80% ethanol. The active fraction was eluted with 60% ethanol. It was subsequently freeze-dried and re-suspended in 1.5 ml of 0.1% TFA (Fraction III). The active component was further purified by high performance liquid chromatography (HPLC).

c) **High performance liquid chromatography (HPLC).** Fraction III (200 µl aliquots) was injected onto a C_{18} reverse phase HPLC column (Waters Delta-Pak,

10 x 250 mm, 10 μm particle size: 300Å pore size: flow rate 1.0 ml/min: mobile phase, 0.1% TFA (A) and 70% acetonitrile in 0.1% TFA (B)) and eluted using a gradient method. The gradient conditions were: 30% solvent B for 3 min; 30% to 45% solvent B in 7 min; 45% to 75% solvent B in 25 min. The eluant was monitored for absorbance at A_{214} and activity was assayed against the indicator strains *E. coli* DH5α and MH1. HPLC-purified colicin was freeze-dried and resuspended in 200 μl of 0.1% TFA (Fraction IV). Colicin activity of all fractions from I to IV was determined by the critical dilution method. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

3.2.4. Tricine-SDS-Polyacrylamide Gel Electrophoresis

The molecular size and purity of HPLC purified colicin Y101 was determined by tricine-SDS-PAGE system. Duplicate samples of HPLC-purified colicin Y101 were applied to tricine-SDS-PAGE gels together with low molecular weight markers (Promega, Madison, WI). Electrophoresis was performed by the method of Schägger & von Jagow (1987). After electrophoresis, the gel was cut into two halves, each of them containing purified colicin Y101. The gel was fixed in 50% methanol for 1 h and washed with distilled water for 1 h. One half of the gel was overlayered with NB soft agar (0.75%) containing a 1% inoculum of *E. coli* DH5α and incubated at 37°C overnight (as described in chapter 2). The visualized zone of inhibition was used to determine the location of colicin Y101. The other half of the gel was stained with Coomassie Brilliant Blue (Bio-Rad Laboratories Ltd., Mississauga, ON).

3.2.5. Mass-spectrometry

The mass of colicin Y101 was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry following the protocol of Rose *et al.* (1999). For crude preparations, the culture supernatant was boiled for 5 min or heated at 75°C for 30 min to inactivate cells. A 0.5 µl volume was spotted onto a probe (stainless steel sample plate), air-dried, washed for 30 s by immersion in sterile distilled water and dried again. A matrix consisting of a saturated solution of sinapinic acid in two parts 0.1% TFA and one part acetonitrile was freshly prepared. Then, 0.5 µl of matrix was spotted onto the sample and air-dried.

For HPLC-purified colicin Y101 or standards, 0.5µl of sample was either spotted and air-dried, followed by spotting of an equal volume of matrix, or mixed with an equal volume of matrix, spotted onto the probe and air-dried. The sample was analyzed in positive ion mode on a linear Bruker Proflex III MALDI-TOF equipped with delayed extraction technology and a 125 cm flight tube (Bruker Analytical Systems, Billerica, MA).

The instrument was calibrated with two points that bracket the mass range of the analyte immediately before collecting data for samples. Angiotensin (MH^+ = 1046.54) and bovine insulin (MH^+ = 5734.56) were used as calibrants (Rose *et al.,* 1999). Fifty spectra of each sample were collected.

3.2.6. DNA isolation and manipulation

Standard techniques were employed for plasmid DNA preparation, restriction endonuclease analysis, ligation, transformation, and electrophoresis (Sambrook *et al.,* 1989). Large- and small-scale plasmid isolation from *E. coli*, DNA manipulation, and cloning were done as described by Sambrook *et al.* (1989). Plasmids were purified by

CsCl density gradient ultracentrifugation. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase (New England Biolabs, Mississauga, ON) and Klenow enzyme (Promega, Madison, WI) were used as recommended by the suppliers. DNA fragments were recovered from agarose gels using either Geneclean II (Bio 101 Inc., La Jolla, CA) or QIAEX II (QIAGEN, Chatsworth, CA). Competent *E. coli* cells were prepared and transformed according to the one-step method of Chung *et al.* (1989).

3.2.7. Molecular cloning of colicin Y101 genes

The plasmid DNA of parental *E. coli* KB101 was transformed into *E. coli* DH5α and MH1 by electroporation and transformants were selected based on immunity to colicin Y101. For selection of transformants, culture supernatant of *E. coli* KB101 was heat-treated at 75°C for 45 min, centrifuged at 11,700 x *g* for 40 min and membrane filtered (PVDF membrane, 0.2 μm pore size, Waters Limited, Mississauga, ON, Canada). Nutrient broth with 3% agar medium was autoclaved and mixed with the same volume of filtered supernatant. Electrotransformed competent *E. coli* cells were streaked onto the surface of the selective plates and incubated at 37°C overnight. Colicin Y101-immune colonies were isolated and examined for colicin activity against non-transformed *E. coli* DH5α by deferred inhibition test. Colicin Y101-positive strains containing pKY1 were subjected to cloning and sequencing of colicin Y101 genes.

With the combination of partial and/or complete digestion by several restriction endonucleases, the size of plasmid pKY1 was determined. Shotgun cloning of either a *Sac*I or a *Hin*dIII partial restriction digest of pKY1 into the compatible site of pUC118 and/or pMG36e (Van de Guchte *et al.,* 1989; Van Belkum and Stiles, 1995) was performed and colonies were screened by designated antibiotic resistance and tested for colicin activity and immunity to colicin Y101 by deferred inhibition test. Partially digested *Hin*dIII fragments of pKY1 were ligated with *Hin*dIII-cut pUC118 while

fragments partially digested with *Sac*I were ligated into the vector plasmid pMG36e. After transforming *E. coli* DH5α separately with each ligation mixture, ampicillin-resistant (pUC118) and erythromycin-resistant (pMG36e) were selected and tested for colicin Y101 production and immunity by the deferred inhibition test.

3.2.8. Nucleotide sequencing analysis of pColY101

The recombinant plasmid DNA pKY9 (Table 3.1) was used as a template. DNA sequencing was performed bi-directional and analyzed in an Applied Biosystems 373A sequencer using fluorescent dideoxy-chain terminators (API Alberta Peptide Institute, University of Alberta, AB). Forward and reverse universal primers for the pUC118 were used for sequencing and specific oligonucleotides were synthesized on an Applied Biosystems 430A peptide synthesizer for sequencing in a primer-walking strategy (API Alberta Peptide Institute, University of Alberta, AB). The DNA Strider program (version 1.2) was used to analyze the nucleotide sequence of pColY101.

3.2.9. Nucleotide sequence accession number

The nucleotide sequence of the 5.0 kb *Hin*dIII-*Sac*I fragment cloned from pKY1, which carries ColY101 structural, immunity and export genes was submitted to GenBank (Los Alamos, NM) and was given the accession number AY536378.

3.3. Results

3.3.1. Purification of colicin Y101

Plasmid DNA was extracted from native colicin Y101 producer strain *E. coli* KB101 and used to electroporate into the colicin-sensitive indicator *E. coli* DH5α. The colicin Y101-positive isolate contained plasmid pKY1 and was designated *E. coli* KY1. Several attempts to purify colicin Y101 using *E. coli* KB101 were unsuccessful because of insufficient activity. Therefore, culture supernatant from the transformant *E. coli* KY1 was used for the purification of colicin Y101. Colicin Y101 produced by *E. coli* KY1 had the same characteristics as *E. coli* KB101. The arbitrary activity units (AU) of the supernatants from *E. coli* KB101 were less than 1600 AU/ml determined by the critical dilution method, while the supernatant from *E. coli* KY1 contained more than 6400 AU/ml.

Four liters of culture supernatant of *E. coli* KY1 (Fraction I) containing a total 2.56 X 10^7 AU was applied to an Amberlite XAD-8 hydrophobic column pre-equilibrated with 0.1% TFA. The colicin was mostly eluted with 60% ethanol and 64% (1.64 X 10^7 AU) of the starting activity was recovered. This fraction was concentrated to 320 ml by rotary evaporation (Fraction II), loaded directly onto a descending SP Sepharose cation exchange column pre-equilibrated with 20 mM sodium acetate buffer (pH 5.0) and eluted with increasing concentrations of NaCl. Most colicin activity was detected in 500 mM NaCl. The eluant was desalted on a Sep-Pak C18 column (Fraction III) and a total of 6.4 x 10^6 AU (25% of starting activity) was recovered from the 70% ethanol fraction. From the first injection of this fraction into the HPLC, a major broad peak was eluted with a HPLC retention time of 25.84 min at about 42.5% acetonitrile concentration (Fig. 3.1A) and contained the majority of the activity; however, lower levels of activity were detected in all subsequent fractions.

This fraction with the majority of the activity was collected, freeze-dried, and re-suspended in 200 µl of 0.1% TFA. 4 separated aliquots of re-suspended sample were further purified under the same conditions. From the second injection, sample was collected at 20 sec time intervals and the highest active fraction was collected. After the same process was repeated, the active single peak appeared at the same position as before and pure colicin Y101 was obtained (Fraction IV, Fig. 3.1B). Approximately 1.5 mg of pure colicin Y101 was obtained representing recovery of 21% of the total activity. Purity of the HPLC-purified colicin Y101 was confirmed by tricine-SDS-PAGE gel electrophoresis.

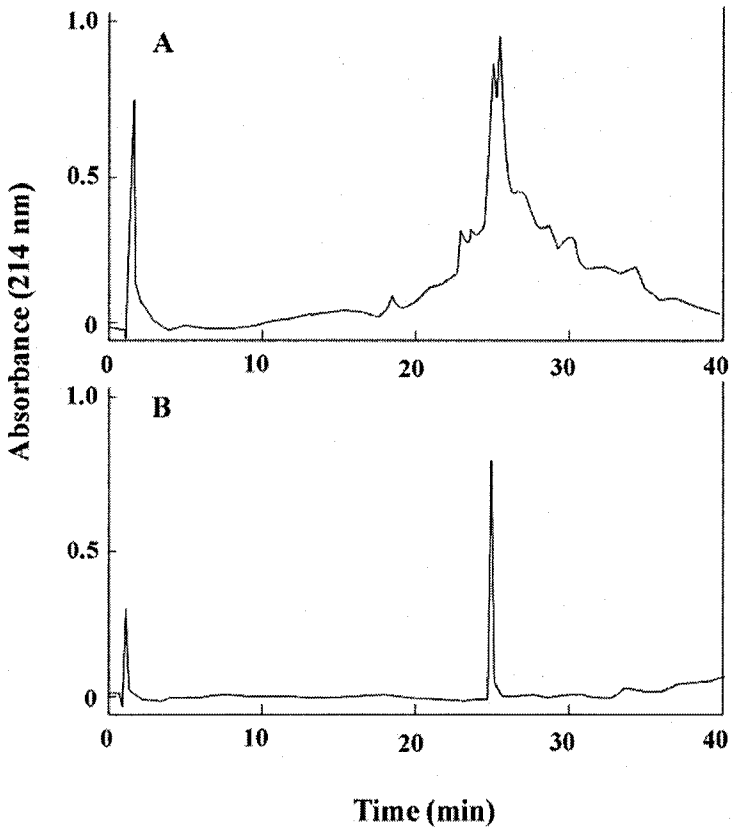


Figure 3.1 Elution pattern of colicin Y101 on reverse-phase HPLC using 0.1% TFA/acetonitrile gradient. A) First injection of sample into HPLC, one major peak was collected, concentrated and re-injected. B) Re-injection of a fraction from A and elution under the same conditions.

3.3.2. Tricine-SDS-PAGE gel electrophoresis

The purified colicin Y101 sample was diluted with equal volumes of 2x sample buffer for tricine-SDS-PAGE and held in a boiling water bath for 4 min before loading onto the gel. The colicin activity in this buffer was confirmed to be active against colicin sensitive strain of *E. coli* DH5α. A single prominent band matched the 8.1 kDa molecular weight marker on the stained gel (Fig. 3.2). The gel overlayered with DH5α developed clear zone of inhibition indicating that the colicin Y101 remained biologically active. The same location of corresponding bands in both stained and overlayered gels was observed (Fig. 3.2). The molecular weight of colicin Y101 and colicin V determined by tricine-SDS-PAGE were almost identical (data not shown).

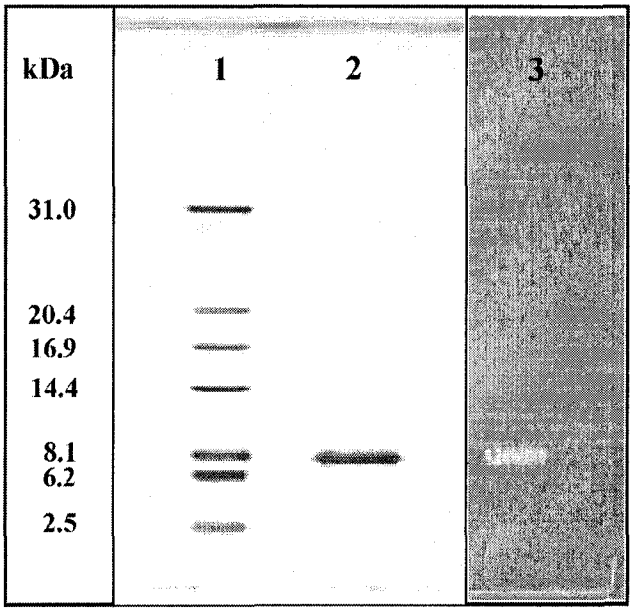


Figure 3.2 Tricine-SDS-PAGE gel of HPLC-purified sample containing colicin Y101: Lane 1, Low molecular weight markers; Lane 2, colicin Y101. Lane 3 was overlayered with indicator to show the zone of inhibition.

3.3.3. Mass spectrometry of purified colicin Y101

Using MALDI-TOF mass spectrometric analysis, a peak with a mass ranging from 8,761 to 8,787 Da was detected from the culture supernatants of *E. coli* KB101 and KY1. Similar mass ranges were detected in each sample from active fractions I to IV of all purification stages (data not shown). The mass of HPLC-purified colicin Y101 shows that colicin Y101 has a mass of 8,776.6 ± 15 Da (Fig. 3.3). ColV has a molecular weight of 8,735.8 Da calculated from the 88 amino acids of the mature peptide and 8,741 ± 17.5 Da analyzed by MALDI-TOF mass spectroscopy (Fath *et al.,* 1994). We detected a compound with a mass of 8,740 ± 15 Da in samples from the culture supernatant and partially purified active fractions of the ColV producer strain *E. coli* MC4100 carrying plasmid pHK22 (see Fath *et al.,* 1992; Gilson *et al.,* 1990). These results indicate that colicin Y101 probably is not identical to ColV.

3.3.4. Restriction analysis and cloning of colicin Y101 genes

With the combination of partial and/or complete digestion by several restriction endonucleases, the size of plasmid pKY1 was determined to be about 120 kb. Among the isolated colonies from the shotgun cloning of either a *Sac*I or a *Hin*dIII partial restriction digest of pKY1 into the compatible site of pUC118 and/or pMG36e, two Col^+ and Imm^+ clones in pMG36e and one Col^+ and Imm^+ clone in pUC118 were isolated. Using single and double digests with *Hin*dIII and *Sac*I, a restriction map of the three inserts was constructed and it was confirmed that they are not identical (Fig. 3.4). One of the pMG36e derivatives containing 22.9-kb *Sac*I fragment was designated pKY5 and the other containing a 14.6-kb *Sac*I fragment was designated pKY7. The pUC118 derivative containing 6.6-kb *Hin*dIII fragment was designated pKY9 (Fig. 3.4).

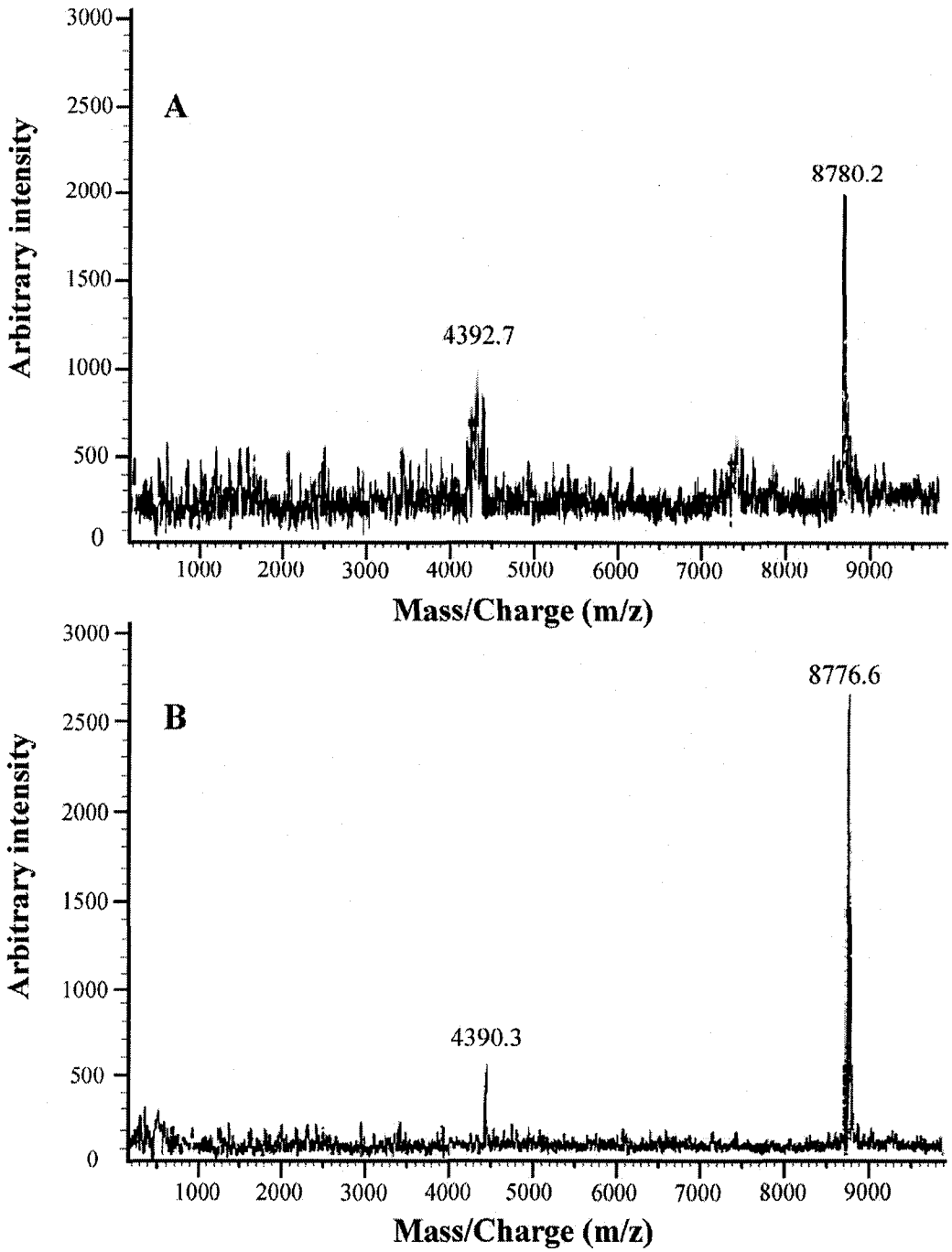
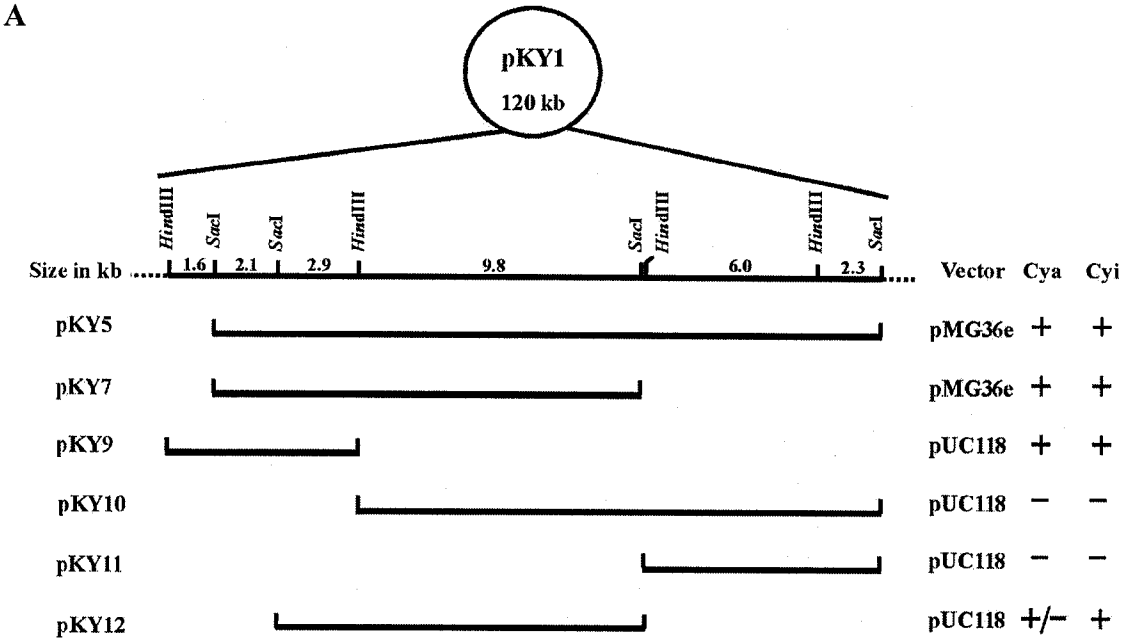


Figure 3.3 Mass spectrometric analysis of purified colicin Y101 by MALDI-TOF mass spectrometry. Mass spectrum obtained (A) from resuspended freeze dried sample of a Sep-Pak C18 column (fraction III), (B) from HPLC-purified colicin Y101 (fraction IV).

A



B

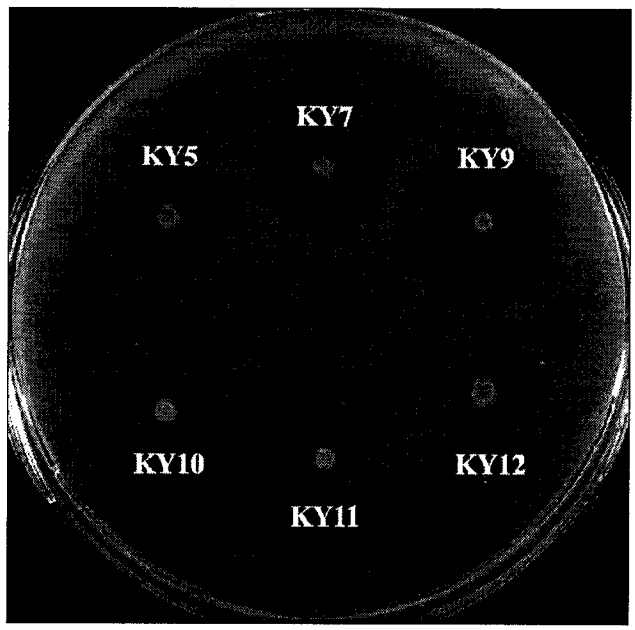


Figure 3.4 (A) Restriction map of colicin Y101 region from plasmid pKY1 and (B) deferred inhibition zone of its derivatives against *E. coli* DH5α.

Further deletions were made in the plasmid pKY5 by cutting with *Hin*dIII and *Sac*I and re-ligating with vector pUC118 and pMG36e.

Cells transformed with plasmids pKY5, pKY7 and pKY9 sharing a region of 5.0-kb *Sac*I-*Hin*dIII segment were Col^+, Imm^+ phenotype (Fig. 3.4). Both the 18.1- and 8.3-kb *Hin*dIII-*Sac*I fragments were cloned from pKY5 into pUC118 resulting pKY10 and pKY11, respectively. *E. coli* DH5α and MH1 transformed with pKY10 or pKY11 were assayed for colicinogenic and immunity properties but it did not confer either characteristic. When both fragments were cloned into pMG36e and the resulting plasmids were transformed into *E. coli* DH5α and MH1, both recombinants were Col^-, Imm^- phenotype (data not shown). Cells with pKY12 containing a 12.6-kb *Sac*I-*Hin*dIII fragment in pUC118 conferred unstably-expressed colicin activity and had full immunity.

3.3.5. Subcloning of pKY9

To produce a functional map of the colicin Y101 genes, further deletion derivatives were inserted in plasmid pUC118 and transformed into *E. coli* DH5α. Their Col and Imm phenotypic recombinants are shown in Fig. 3.5. The region of 5.0-kb *Sac*I-*Hin*dIII fragment was shown to confer full colicin production and immunity.

pKY37 containing the 3.7-kb *Hin*dIII-*Sac*I fragment did not confer colicin production and immunity to the cells. The 2.9-kb *Hin*dIII-*Sac*I fragment in pKY29 expressed partial colicin activity and full immunity. The 2.1-kb *Sac*I fragment was deleted from pKY9 and the remaining fragments were re-ligated resulting in pKY45. This clone also expressed partial colicin activity and full immunity. Plasmid pKY50 containing the 5.0-kb *Hin*dIII-*Sac*I fragment produced full colicin activity and immunity. The zone of inhibition generated by pKY50 was larger than that observed with the wild type *E. coli* KB101 and the transformant *E. coli* DH5α (KY1) carrying the native colicin Y101 plasmid pKY1 (Fig. 3.6). The same results were achieved using pMG36e as a

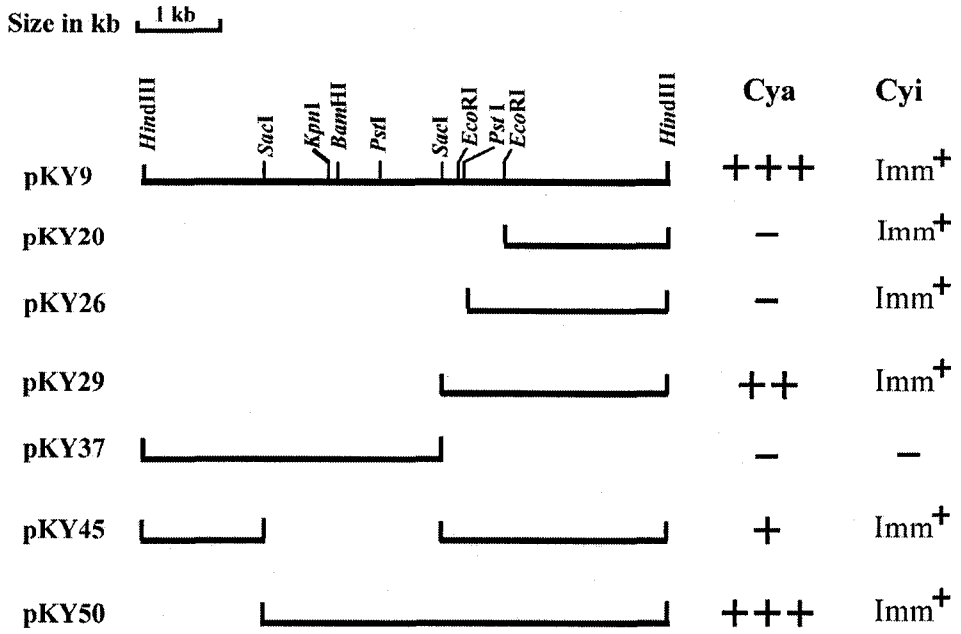


Figure 3.5 Restriction map of pKY9 carrying the 6.6 kb *Hin*dIII-*Sac*I fragment of pKY9 and its derivatives. The expression vector DNA was pUC118 for all derivatives. +++: >25 mm, ++: 10-15 mm; +: <10 mm, and -: negative

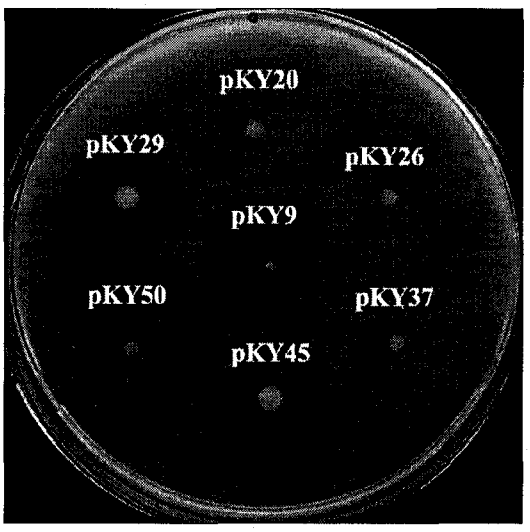


Figure 3.6 Deferred inhibition test with *E. coli* DH5α containing plasmid derivatives from pKY9 against non-transformed *E. coli* DH5α .

vector and *E. coli* MH1 as a host strain (data not shown).

Two other constructs containing 2.6 and 2.0 kb *Eco*RI-*Hin*dIII fragments did not confer activity but expressed full immunity. These results indicate that the gene(s) responsible for immunity of colicin Y101 are located on a 2.0 kb *Hin*dIII-*Eco*RI fragment while the 5.0 kb *Hin*dIII-*Sac*I fragment is responsible for colicin activity.

3.3.6. Nucleotide sequence analysis and amino acid homology

The 5.0 kb *Hin*dIII-*Sac*I fragment in pKY50 was completely sequenced in both directions using its deleted derivatives and specific synthetic primers. The nucleotide sequence of the 4,970-bp *Hin*dIII-*Sac*I fragment is shown in Fig. 3.7. Analysis of the nucleotide sequence identified four possible ORFs in two converging operons, one with two ORFs relating to export of colicin Y101 and the other with two ORFs relating to structure and immunity.

The amino acid analysis revealed that two ORFs relating to colicin Y101 export are similar or identical to *cvaA* and *cvaB* for ColV translocation. There were only four differences in amino acids compared with CvaB at positions 305, 486, 496, and 566, in which the amino acids for CyaB of the colicin Y101 were substituted as follows: Cys→Phe, Asp→His, Gly→Asp, and His→Gln, respectively. These two genes, *cyaA* and *cyaB* overlap each other in an identical manner to those in ColV operon (Fig. 3.7).

The genes encoding colicin Y101 structure (*cya*) and immunity (*cyi*) on the other opposite strand of the DNA were identified. These genes are co-transcribed in the opposite direction to the genes for the export proteins. The initiation codon of the structural gene (*cya*) overlapped the end of the immunity gene (*cyi*). Amino acid analysis revealed very high homology of these ORFs with ColV structural (*cvaC*) and immunity (*cvi*) genes. Colicin Y101 can therefore be considered to be a natural variant of ColV with differences involving replacements: Ile^{49} to Val and Ala^{86} to Asp.

Sac I
GAGCTCTTGTCCAGTGTATCACCACTACCATGGGGTCATATCTCGCTCACTGGAGATTACCTGTGGAACGAAATTGACCA 80
TCCCCTTGAACGATACAGGCCACTTCGTACTAATCGCTTCAATCCAAAGAACTTCACTTTTCCTTAGCGTGTGTTATCGC 160
AGAAATGCCCACGGAACCCCGAGTATGCCTGCCCTTCCCTAGAGAATCCTGCCAGGTTTGCCACACTGATATATCTTGAC 240
TTTATGTAAACAATATGACACCTTAACATGATAATGATTACCATTATCTTTTAATATACAGAGAAACTAGGAAATAGATG 320
AATGAGTTATGTTACTTTAATATTCTCTGACAATAACCTAAATCAGTTAGATTATTGTCATTTAATAAATAATGACATTC 400
TTTCATCATAAATAAAAAGACTATTGTTTATAATATTGTTCTCAGCATTATATGATTATTTATCCTGATAACTCTCCTAT 480
-35 -10
GTTGTATG**TTTATA**TGATTTTCCTTGAAACA**TATAAT**GCAAATTTTCGATTTATTTTCCATCATTAATCCAGATAAACAA 560

RBS cyaA start →
CAAACTAATAGTATGCAAGGAGACATTATTTGTTTCGCCAGGATGCTTT**AGAAAA**CAGAAAA**ATG**AAGTGGCAGGGACGG 640
M K W Q G R

GCAATATTACTTCCCGGAATACCACTGTGGTTAATCATGCTGGGAAGCATTGTGTTTATTACGGCATTTCTGATGTTCAT 720
A I L L P G I P L W L I M L G S I V F I T A F L M F I
Kpn I
TATTGTT**GGTACC**TATAGCCGCCGTGTTAATGTCAGTGGTGAGGTCACAACCTGGCCAAGAGCTGTCAATATATATTCAG 800
I V G T Y S R R V N V S G E V T T W P R A V N I Y S G
*Bam*H I
GTGTACAGGGATTTGTTGTCAGGCAGTTTGTTCATGAAGGGCAGTTGATAAAAAAAGG**GGATCC**TGTTTATCTGATTGAC 880
V Q G F V V R Q F V H E G Q L I K K G D P V Y L I D

ATCAGTAAAAGTACACGCAATGGTATTGTCACTGATAATCATCGCCGGGATATAGAAAACCAGCTGGTTCGTGTGGACAA 960
I S K S T R N G I V T D N H R R D I E N Q L V R V D N

CATTATTTCCCGTCTGGAAGAAAGTAAAAAAATAACGCTAGATACCCTGGAAAAACAACGTCTGCAATACACAGATGCGT 1040
I I S R L E E S K K I T L D T L E K Q R L Q Y T D A F

TCCGTCGCTCATCAGACATTATACAGCGTGCAGAGGAAGGGATAAAAATAATGAAAAATAATATGGAGAATTACAGATAC 1120
R R S S D I I Q R A E E G I K I M K N N M E N Y R Y

TATCAGTCAAAAGGACTGATTAATAAAGATCAATTAACTAACCAAGTTGCATTATATTATCAACAACAAAACAACCTTCT 1200
Y Q S K G L I N K D Q L T N Q V A L Y Y Q Q Q N N L L

CAGTCTGAGCGGACAAAATGAACAAAATGCCCTGCAAATAACCACTCTGGAGAGTCAGATTCAGACTCAGGCAGCAGATT 1280
S L S G Q N E Q N A L Q I T T L E S Q I Q T Q A A D F

TTGATAATCGTATCTATCAGATGGAACTGCAACGACTCGAATTGCAGAAAGAACTGGTTAACACTGATGTGGAAGGCGAA 1360
D N R I Y Q M E L Q R L E L Q K E L V N T D V E G E

ATCATTATCCGGGCGTTGTCTGACGGGAAAGTTGACTCCCTGAGTGTCACTGTAGGGCAAATGGTCAATACCGGAGACAG 1440
I I I R A L S D G K V D S L S V T V G Q M V N T G D S

Pst I
CCTT**CTGCAG**GTTATTCCTGAGAACATTGAAAACTATTATCTTATTCTCTGGGTCCCGAATGATGCTGTTCCTTATATTT 1520
L L Q V I P E N I E N Y Y L I L W V P N D A V P Y I S

CTGCTGGTGACAAAGTGAATATTCGTTATGAAGCCTTCCCCTCAGAAAAATTTGGGCAGTTCTCTGCTACGGTTAAAACT 1600
A G D K V N I R Y E A F P S E K F G Q F S A T V K T

Figure 3.7 The complete single stranded nucleotide sequence of pKY50 carrying the colicin Y101 operon. The numbers on the right refer to the nucleotide position. The translation products of *cyaA*, *cyaB*, *cya*, and *cyi* are shown below the nucleotide sequence. The double-glycine-type leader peptide is shown in bold letters and its cleavage site is indicated with a vertical arrow. The restriction endonuclease sites are shown in bold letters. The two possible in-frame ORFs and the promoter regions and potential start sites are marked in bold letters.

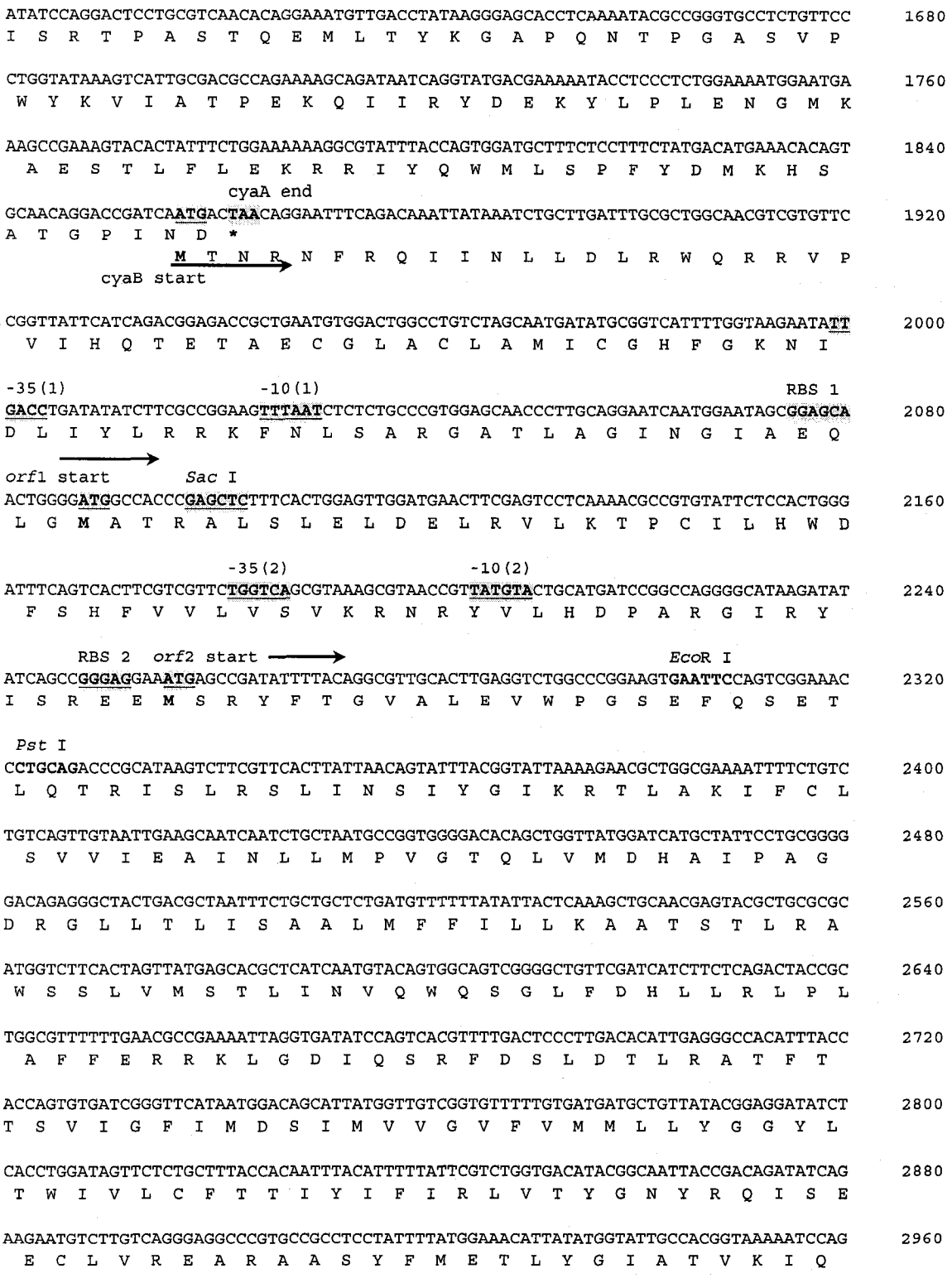


Figure 3.7 Continuation.

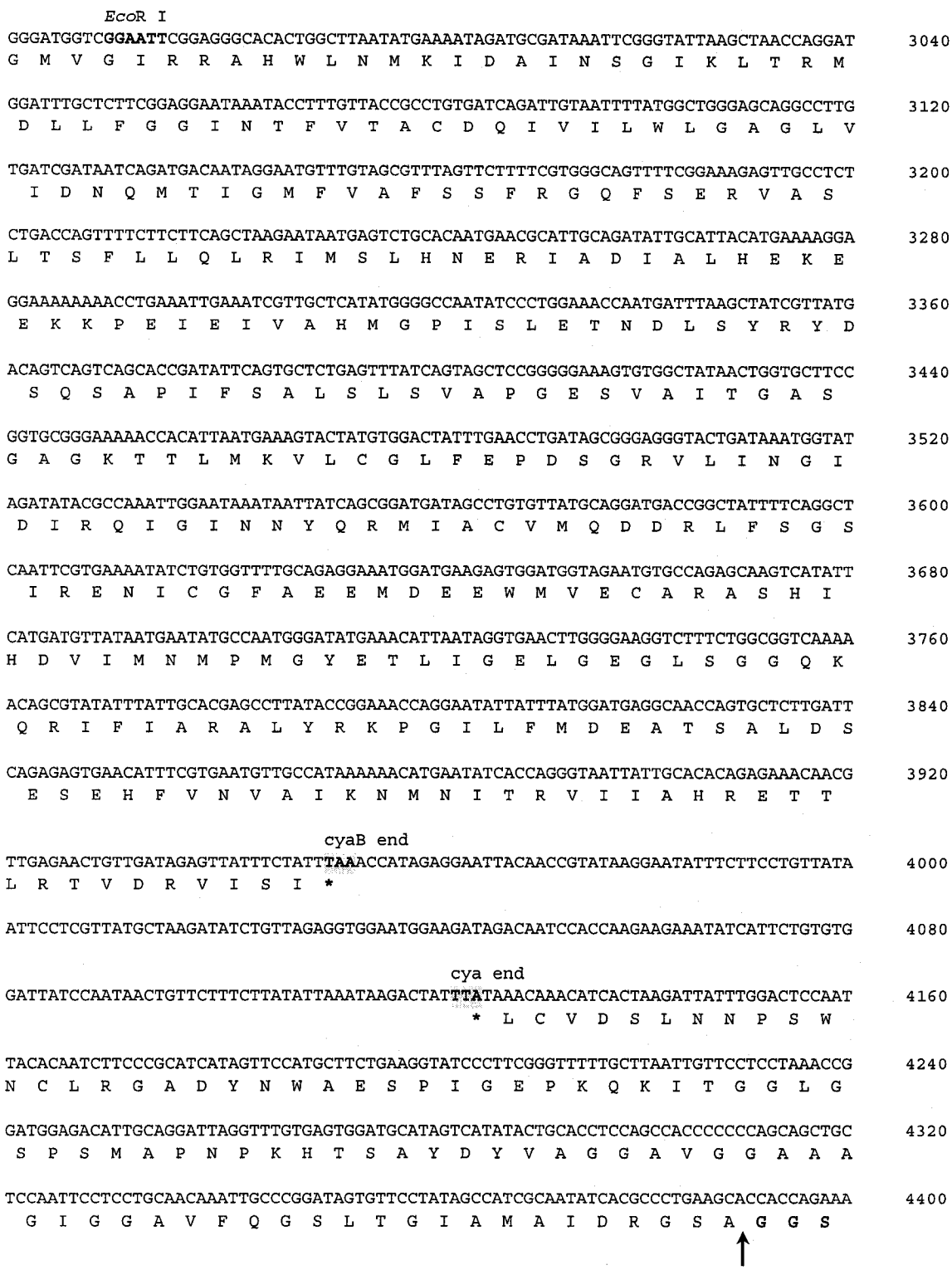


Figure 3.7 Continuation

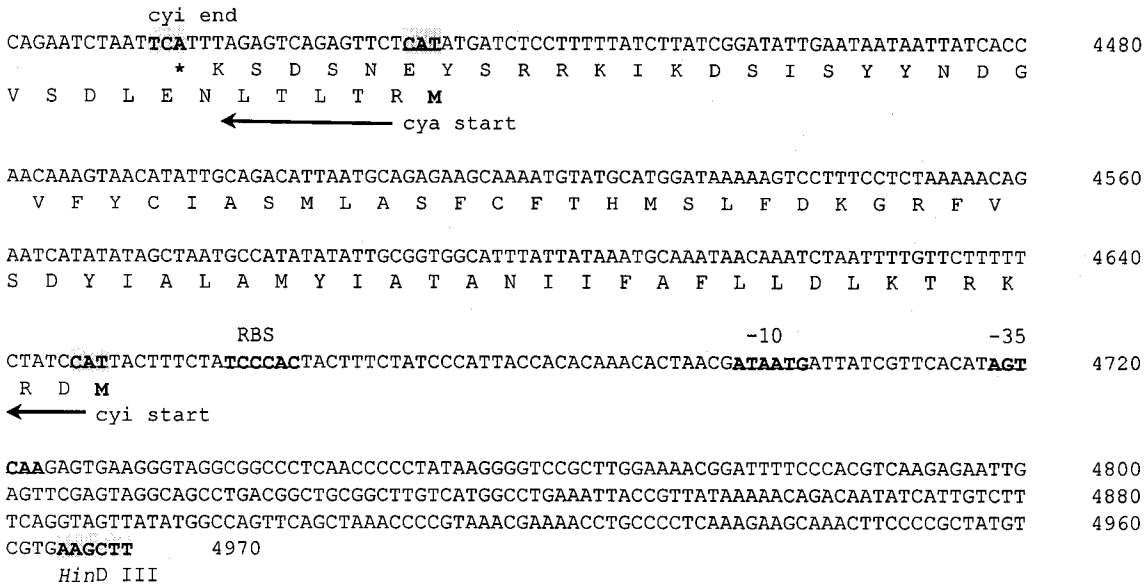


Figure 3.7 Continuation

Two amino acid differences were also observed in the colicin Y101 immunity protein: Glu^{9}→Asp and Ile^{23}→Met. Similar to ColV, the colicin Y101 structural gene encodes a 103-amino-acid pre-peptide containing a 15-amino-acid N-terminal leader peptide of double glycine type that is identical to the ColV leader peptide. The calculated molecular mass of mature colicin Y101 is 8,765.8 Da compared with the analyzed average MALDI-TOF mass of 8,776.6 Da.

3.3.7. The possibility of two in-frame *orf*s in *cyaB*

Two possible in-frame start sites seem to be located downstream of *cyaB* initiation codon (in positions 78 and 134). A probable ribosomal binding site (RBS) for the first putative ORF (*orf*1) was GGAGA and located nine bases upstream of the initiation codon (ATG). The possible promoter sequences for −35 and −10 indicated in Fig. 3.7 were TTGACC and TTTAAT, respectively. *Orf*1 could encode an in-frame

protein of 621 amino acids. Immediately downstream of *orf*1 was the second possible in-frame ORF (*orf*2) that could contain 566 codons. A probable RBS for *orf*2 (GGGAG) and promoter region for -35 (TGGTCA) and -10 (TATGTA) are indicated in Fig. 3.7. These two putative *orf*s appeared to encode a truncated *cyaB* with ATP-binding cassette (ABC) domains in its C-terminus. Interestingly, we observed zones of inhibition from $CyaA^-/B^-$ (pKY29 and 45) genotypes. Both pKY29 and 45 contain *orf*2 (Fig. 3.5, 3.6, and 3.7). In contrast, pKY 20 and 26 do not produce zones of inhibition but they are immune to ColY 101. These two constructs contain deletions of *orf*2.

3.4. Discussion

Even though colicin activity was detected by the spot-on-lawn technique the amount of colicin Y101 produced by *E. coli* KB101 was very low. This indicates that colicin Y101 probably has a high specific activity for the indicator strains. When a SDS-PAGE gel was stained with Coomasie Brilliant Blue, a designated protein band for colicin Y101 was not detected while the overlayered gel gave a clear zone of inhibition (data not shown). Successful purification was achieved using the transformant *E. coli* KY1. The approximate molecular weight (MW) of colicin Y101 by tricine-SDS-PAGE was 8.1 kDa. Several trials to measure its MW using the methods similar to or modified from those described by Laemmli (1970) were not successful because of difficulties in clearly separating the low-molecular weight marker proteins (LMW; less than 10 kDa) (data not shown). An apparent single band detected in the HPLC-purified sample was slightly larger than the molecular standard of 6.5 kDa, aprotinin. However, the resolution obtained from tricine-SDS-PAGE system described by Schägger and von Jagow (1987) was clear enough to separate the LMW standard proteins, myoglobin F1, F2 and F3 with molecular weights of 8.1, 6.5 and 2.5 kDa, respectively. From these data, colicin Y101 was matched with 8.1 kDa, myoglobin F1. As described in chapter 2, colicin Y101 is highly stable under a wide range of chemical and physical conditions. Colicin Y101

retained its activity under the acidic conditions and when treated with ethanol and methanol during purification. Boiling in SDS-buffer did not affect the activity of colicin Y101.

The genetic determinants of colicin Y101 were identified from a 5.0-kb *Hin*dIII-*Sac*I fragment of the 120-kb native plasmid pKY1. Recombinant clones containing the 14.6-kb *Sac*I fragment in pMG36e and 6.6-kb *Hin*dIII fragment in pUC118 conferred colicin production and immunity. Restriction analysis of these clones revealed the location of genetic determinant for colicin Y101 production, immunity and export. A 5.0-kb *Hin*dIII-*Sac*I fragment from pKY9 resulted in full production and immunity of colicin Y101. The nucleotide sequence of this 4,970-bp fragment revealed four ORFs organized in two converging operons that encodes colicin Y101 and show extensive homology with the cloned region of pColV-B188 and pColV-K30 for ColV (Frick *et al.,* 1982; Gilson *et al.,* 1987). Search for amino acid homology revealed extensive identity between ColY101 and ColV proteins. The genes involved in colicin Y101 export had 100% identity to CvaA and 99.4% identity to CvaB. Similarly, colicin Y101 structural and immunity genes show 98% and 97% identity to ColV structural and immunity genes, respectively. Therefore, colicin Y101 may be a natural variant of ColV.

Colicin Y101 is first translated as a 103-amino-acid precursor protein containing 15-amino-acid double-glycine-type leader peptide. The N-terminal 15 amino acids of Col Y101 are identical to the ColV leader peptide. Gilson *et al.* (1990) reported that the structural gene (*cvaC*) for ColV encodes 103 amino acid residues and that the secreted translation product of *cvaC* is processed to remove the N-terminal 15 amino acids, yielding the mature peptide consisting of 88 amino acids. This N-terminal extension with specific Gly-Gly↓ cleavage site is similar to N-terminal extensions reported for class IIa bacteriocins of Gram-positive lactic acid bacteria. This so-called double-glycine-type leader peptide is associated with a dedicated bacteriocin export system (Fath *et al.,* 1994; Klaenhammer, 1993; Nes *et al.,* 1996). CvaA and CvaB are known to form a dedicated

transport system and the outer membrane protein TolC is required for export of ColV.

Based on the similarities between ColV and ColY101, it may be assumed that a divergent genetic evolution occurred by natural mutagenesis. As demonstrated in chapter 2, the difference in the antibacterial spectrum between ColY101 and ColV is not great, but the inhibition profile of ColV is included in that of ColY101. The zones of inhibition produced by ColY101 are larger than those of ColV (data not shown) indicating the possibility that colicin Y101 is produced in higher concentration or that it has a higher specific activity than ColV. Several cases of natural bacteriocin variants produced by Gram-positive bacteria have been reported. Nisin A differs from nisin Z by only one amino acid (Mulder *et al.,* 1991). Although leucocin A-UAL187, B-TA11a, and C-LA7a produced by strains of *Leuconostoc* and *Weissella* are identical bacteriocins (Hastings *et al.,* 1991, 1994, 1996), mesentericin Y105 produced by *Leuconostoc mesenteroides* has two amino acids that differ from leucocin A (Hastings *et al.*, 1991; Héchard *et al.*, 1992).

The immunity protein of ColY101 consists of 78 amino acids and has two amino acid residues in positions 9 and 23 that differ from ColV. These two amino acid replacements did not confer changes in immunity; however, the ColV producer carrying plasmid pHK22 is not fully immune to the ColY101 producer carrying plasmid pKY50. This may be due to the over-production or higher specific activity of ColY101. According to Fath *et al.* (1992) and Gilson *et al.* (1990), the ColV immunity protein (Cvi) spans the inner membrane, localizing its N- and C-termini in the cytoplasmic space. One of replaced amino acids (Asp in position 9) is located in the cytoplasmic space and the other one (Met in position 23) is located in membrane-spanning domain.

It was not expected to observe a clear zone of inhibition produced by the $CyaA^-$ B^- genotype that is carrying plasmid pKY29 and pKY45 (Figs. 3.5, 3.6, and 3.7) because the CvaAB/TolC protein complex is essential for ColV secretion and no detectable ColV production was found in either $CvaA^-$ or $CvaB^-$ in the presence of TolC outer membrane protein (Fath *et al.,* 1993; Skvirsky, 1995; Zhang et al., 1995; Zhong et al., 1996). The

cells transformed with pKY29 and pKY45 containing a deletion in the genes for export should not confer colicin activity. In our study, we observed clear zones of inhibition from the transformants carrying plasmid pKY29 and pKY45 ($CyaA^-B^-$) (Figs. 3.4, 3.5, 3.6, and 3.7).

Interestingly, two possible promoter sequences were found immediately downstream of CyaB (Fig. 3.7). The ABC transporter, CvaB of ColV, has six potential transmembrane domains located in the inner membrane with a typical ATP-binding cassette in a C-terminal cytoplasmic region (Fath *et al.,* 1992). CvaB requires CvaA to connect to the TolC in the outer membrane to facilitate export of ColV. If the truncated in-frame protein CyaB is translated that contains cytoplasmic ATP-binding domain located in the C-terminus, functions to cleave the leader peptide yielding the mature peptide and supports its transport across the inner membrane, the question arises how this mature peptide crosses the periplasmic space and outer membrane without CyaA.

As described earlier, ColV shares characteristics with bacteriocins of Gram-positive bacteria especially several non-lantibiotic bacteriocins. For bacteriocins in this group, a dedicated membrane protein complex is comprised of a two-component ATP-binding cassette (ABC) family of proteins, which are involved in bacteriocin export (Van Belkum and Stiles, 1995; Franke *et al.*, 1996; Higgins, 1992, Higgins *et al.*, 1996). Colicin V requires the chromosomally encoded outer membrane protein TolC for its release in addition to this protein complex. Using the similarity of the N-terminal leader sequence, successful heterologous expression of bacteriocins has been achieved (Van Belkum *et al.*, 1997; Franz, 1997; McCormick et al., 1998, 1999). As a natural variant of ColV, the N-terminal leader peptide of ColY101 is identical to the N-terminal leader of ColV suggesting the potential for the heterologous expression of ColY101 in lactic acid bacteria. Chapter 4 deals with colicin Y101 expression in lactic acid bacteria using heterologous expression, as well as multiple colicin expression. We plan to take advantage of the enhanced activity profile of colicin Y101.

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CHAPTER 4

EVALUATION OF COLICIN Y101 SECRETION SYSTEM AND HETEROLOGOUS EXPRESSION IN LACTIC ACID BACTERIA

4.1. Introduction

Natural *Escherichia coli* populations are generally known to carry colicin plasmids that encode antibacterial proteins or peptides, so-called colicins, that kill closely related bacteria competing in the same environment (Braun *et al.*, 1994; Luria and Suit, 1987). Colicins are released from the cell by a mechanism that differs markedly from the typical method of protein release from *E. coli*. In most cases of bacterial proteins, the cleavable N-terminal extension, so-called signal sequence of proteins (peptides) makes the proteins recognizable by the specific general secretion machinery (*Sec*-pathway) and mediates the release of proteins across the cytoplasmic membrane (Pugsley, 1993). Release of colicins is regulated either by a semi-specific or by non-specific process that is accompanied with the release of other cytoplasmic and periplasmic molecules. Release of most colicins is facilitated by so-called lysis proteins that cause simultaneous lysis and death of the cells (Braun *et al.*, 1994; Cavard *et al.*, 1985; Lakey, 1994). The secretion of other colicins that do not contain the lysis gene products is less efficient and the secretion mechanism remains unclear, but it seems to be due to an accumulation of colicin in the cytoplasm that causes membrane damage and cell death (Pugsley, 1984a,b).

Unlike most colicins, the secretion of colicin V is not induced by 'SOS' response nor does it require a lysis protein. Colicin V is encoded as a precursor peptide of 103 amino acids that is processed to remove its 15 N-terminal amino acids (leader peptide) at a double glycine cleavage site that is typical of class II bacteriocins that are found in

many lactic acid bacteria. The mature peptide of 88 amino acids is exported from the cell by a set of dedicated transport proteins consisting of CvaA and CvaB and an outer membrane protein, TolC (Gilson *et al.*, 1990; Håvarstein *et al.*, 1994). CvaB contains an ATP-binding domain in its C-terminus that is homologous to other members of the ATP-binding (ABC) superfamily of export proteins. CvaA is an accessory protein for export of colicin V that belongs to the membrane fusion protein (MFP) family and interacts with CvaB and TolC. TolC is a chromosomally-encoded outer membrane protein that connects the inner and outer membranes to form a dedicated export complex (Fath *et al.*, 1992; Hwang *et al.*, 1997).

Many class II bacteriocins from Gram-positive lactic acid bacteria are produced as precursors with an N-terminal leader peptide of 18 to 24 amino acids containing a conserved double glycine processing site that is removed during the secretion process (Håvarstein *et al.*, 1995; Van Belkum *et al.*, 1997). The secretion of bacteriocins by this group of bacteria requires a dedicated export system formed by two membrane-bound proteins: an ABC transporter protein and an accessory protein. The ABC transporter protein is responsible for cleavage of the N-terminal leader peptide during processing and export of the prepeptide from the cell. The function of the accessory protein is unclear for bacteriocins of Gram-positive bacteria. The double glycine type N-terminal leader peptide of colicin V and class II bacteriocins of lactic acid bacteria share high sequence similarity with a conserved $gly^{-2}gly^{-1}$ Xaa^{+1} cleavage site (Fath and Kolter, 1993; Håvarstein *et al.*, 1994, 1995).

There has been intensive study of the potential to apply bacteriocins and colicins in systems for food preservation. The most promising approach in our lab was the construction of heterologous expression systems using lactic acid bacteria to overcome the limitation of the narrow activity spectrum of many bacteriocins. Van Belkum *et al.* (1997) showed that colicin V could be secreted in *Lactococcus lactis* using the

lactococcin A dedicated export proteins. In addition, the leader peptide of leucocin A, lactococcin A or colicin V could be used to export mature divergicin A that normally is produced by *Carnobacterium divergens* LV13 by the signal sequence dependent *sec*-pathway. It was also shown that the signal peptide of divergicin A could direct the secretion of carnobacteriocin B2 (McCormick *et al.,* 1996) and enterocin B (Franz *et al.,* 1997) that have dedicated export systems. Replacement of the colicin V leader peptide with a signal peptide of divergicin A could also direct colicin V export in lactic acid bacteria (McCormick *et al.,* 1999).

We previously identified colicin Y101 from the *E. coli* KB101 isolated from a beef product. Nucleotide analysis revealed that colicin Y101 was a natural variant of colicin V with differences in only two amino acids and a leader peptide that is identical to that of colicin V. Colicin Y101 exhibited strong inhibitory activity against *E. coli* O157:H7, *Salmonella, Shigella,* and *Klebsiella* spp. This small colicin is stable in chemically and physically harsh conditions. The overall goal of our studies is to develop multiple bacteriocin expression systems in lactic acid bacteria to ensure food safety. The specific objective of this study was to evaluate heterologous expression of colicin Y101 in lactic acid bacteria and the inhibitory activity of colicin Y101 producing lactic acid bacteria against *E. coli* O157:H7 and *Salmonella.* Colicin Y101-producing lactic acid bacteria could have great potential to control Gram-negative spoilage and pathogenic organisms in food fermentation and preservation. This study presents: (1) the analytical study of colicin Y101 and colicin V production in the absence of their dedicated export proteins; and (2) the comparison of ColV and ColY101 activity using dedicated transport pathway and the general *sec*-pathway.

4.2. Materials and Methods

4.2.1. Bacterial strains and media

Bacterial strains and plasmids used in this study are listed in Table 4.1. *E. coli* strains used in this study were DH5α (BRL Life Technologies, Rockville, MD) and MH1 (Casadaban and Cohen, 1980). They were grown on a rotary shaker at 250 rpm in Nutrient broth or Luria-Bertani broth (Difco, BD Diagnostics, Sparks, MD) at 37°C unless otherwise indicated. Carnobacteria were grown in APT broth (Difco) at 25°C without agitation. *L. lactis* was grown in GM17 broth (Terzaghi and Sandine, 1975) at 30°C without agitation. Agar plates were made by addition of 1.5% (wt/vol) agar to designated broth media. Soft agar was made for agar overlayers by addition of 0.75% (wt/vol) agar to designated broth media. Antibiotics were added as selective agents when required: ampicillin 150 μg/ml, chloramphenicol 30 μg/ml, kanamycin 50 μg/ml and erythromycin 200 μg/ml for *E. coli* and erythromycin 5 μg/ml for carnobacteria. Stock cultures were made in the same medium that was used for bacterial culture with 15% glycerol (vol/vol) added and stored at –80 °C.

4.2.2. Colicin activity tests

Deferred inhibition tests were done as described by Ahn and Stiles (1990) to monitor colicin activity of both parental and recombinant strains. Colicin activity assays were done by the critical dilution method as described in chapter 2. *E. coli* DH5α and MH1 were used as the indicator strains, unless otherwise stated, and inoculated (1%) into the appropriate soft (0.75%) agar medium. To confirm the proteinaceous nature of the inhibitory substance (colicin), pronase E was applied adjacent to the expected zone of

inhibition.

Table 4.1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains:		
E. coli DH5α	F-Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1*hsd*R17 (r_k^- m_k^+) *pho*A *sup*E44 λ-*thi*-1*gyr*A96 *rel*A1, colicin sensitive	BRL Life Technology Inc.
E. coli MH1	MC1061 derivative; *ara*D139 *lac*X74 *gal*U *gal*K *hsr* *hsm*$^+$ *str*A colicin sensitive	Casadaban and Cohen (1980)
C. divergens LV13	Contains pCD3.4, *dvn*, *dvi*	Worobo *et al.* (1995)
C. maltaromaticum LV17C	Plasmidless	Ahn and Stiles (1990)
C. maltaromaticum UAL26	Plasmidless	Our collection
L. lactis IL1403	Plasmidless	Chopin *et al.* (1994)
Plasmids:		
pACYC184	Cm^r, Tc^r, 4.2 kb	Chang and Cohen (1978)
pHK22	pACYC184 derivative containing 9.4 kb colicin V gene cluster, *cvaA*, *cvaB*, *cvaC*, *cvi*, Cm^r, 13.6 kb	Gilson *et al.* (1987)
pMG36e	Expression vector, Em^r, 3.6 kb	Van de Guchte *et al.*, (1989)
pUC118	*lacZ'*, Amp^r, 3.2 kb	Vieira and Messing (1987)
pRW19e	514-bp *Eco*RV-*Acc*I fragment cloned in pMG36e; *dvn*, *dvi*, Em^r, 4.1 kb	McCormick *et al.* (1996)
pJKM37 (pDSV1)	295-bp *Hin*dIII-*Kpn*I PCR product from pHK22 cloned in pRW19e, *dvn-cvaC*, Em^r, 4.0 kb	McCormick *et al.* (1999)
pKV72	pUC118 containing 7.2 kb *Hin*dIII-*Bgl*II fragment from pHK22; ColV$^+$, Amp^r	This study
pKV72-1	pACYC184 containing 7.2 kb *Hin*dIII-*Bgl*II fragment from pHK22; ColV$^+$, Cm^r	This study

pKY1	ColY101$^+$, 120 kb	This study
pKY9	pUC118 containing 6.6 kb *Hin*dIII fragment from pKY1; ColY101$^+$, Amp^r	This study
pKY9-1	pACYC184 containing 6.6 kb *Hin*dIII fragment from pKY1; ColY101$^+$, Cm^r	This study
pKY29	pUC118 containing 2.9 kb *Hin*dIII-*Sac*I fragment from pKY9; *cya*, *cyi*, Amp^r	This study
pKY50	pUC118 containing 5.0 kb *Hin*dIII-*Sac*I fragment from pKY9; ColY101$^+$, Amp^r	This study
pDSY1	295-bp *Hin*dIII-*Kpn*I PCR product from pKY29 cloned in pUC118, Amp^r, 3.5 kb	This study
pDSY29	295-bp *Hin*dIII-*Kpn*I PCR product from pDSY1 cloned in pRW19e, *dvn-cya*, Em^r, 4.0 kb	This study

[a] *dvn*, divergicin A structural gene; *dvi*, divergicin A immunity gene; *cvaA/B*; colicin V export gene; *cvaC*, colicin V structural gene; *cvi*, colicin V immunity gene; *cya*, colicin Y101 structural gene; *cyi*, colicin Y101 immunity gene; *dvn-cvaC*, divergicin A signal peptide fused to colicin V structural gene; *dvn-cya*, divergicin A signal peptide fused to colicin Y101 structural gene; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tc^r, tetracycline resistant.

4.2.3. DNA isolation, manipulation and sequence analysis

Small and large-scale plasmid DNA isolations from *E. coli* were done as described by Sambrook *et al.* (1989) and the methods according to Van Belkum and Stiles (1995) and Worobo *et al.* (1995) were applied for DNA isolation from lactic acid bacteria. Standard methods were used for restriction enzyme digestions, ligations and gel electrophoresis. Restriction enzymes, T4 DNA ligase (New England Biolabs, Mississauga, ON) and Klenow enzyme (Promega, Madison, WI) were used as recommended by the suppliers. Competent *E. coli* were prepared and transformed according to the one-step method of Chung *et al.* (1989). Transformation of carnobacteria was performed by the method according to Worobo *et al.* (1995). Plasmids were purified by CsCl density gradient ultracentrifugation and DNA fragments were recovered from agarose gel using either Geneclean II (Bio 101 Inc., La Jolla, CA) or QIAEX II (QIAGEN, Chatsworth, CA). DNA was sequenced by Taq DyeDeoxy Cycle sequencing (Applied Biosystems 373A DNA sequencer, Foster City, CA). Nucleotide sequences were determined bi-directionally in pUC118 using universal primers.

4.2.4. PCR amplification

The divergicin A signal peptide::colicin Y101 fusion construct was created using two PCR primers described by McCormick *et al.* (1999). The primer JMc12 (5'-CCCAAGCTTCTGCTGCTTCAGGGCGTGATATT-3') contained a *Hin*dIII restriction site (underlined) and the following 5 nucleotides (CTGCT), which regenerate the carboxy-terminus of the divergicin A signal peptide (McCormick *et al.,* 1996; Worobo *et al.,* 1995). The 18 nucleotides next to the CTGCT nucleotides encode the amino terminus of mature colicin Y101 structure. The reverse primer JMc27 (5'-

CCCGGTACCACTATTTATAAACAAACATCAC-3') contains a *Kpn*I restriction site (underlined) and it is complementary to the 3' end of the colicin Y101 structural gene. DNA was amplified with *Taq* DNA polymerase (Perkin Elmer, Foster City, CA) in 100 µl using a temperature cycler (OmniGene, Intersciences Inc., Markham, ON). Using pKY29 as template, DNA was amplified in 36 cycles (denaturation, 93°C, 1 min; annealing, 48°C, 1 min; extension, 75°C, 2 min) followed by a final extension step at 75°C for 5 min. The PCR products were cloned into pUC118 for sequencing and to confirm the fidelity of the reactions.

As described in chapter 3, colicin Y101 is a natural variant of colicin V with differences in two amino acids. To compare the activity of colicins V and Y101, the same strategy of McCormick *et al.,* (1999) was used to create a divergicin A signal peptide::colicin V structure fusion construct except that pHK22 was used as the template for the reaction (Gilson *et al.,* 1990; McCormick *et al.,* 1999).

4.2.5. Localization and expression of the colicin Y101 in lactic acid bacteria

C. maltaromaticum UAL26 was used as host strain for the heterologous expression of colicin Y101 unless otherwise stated. A 295-bp fragment that contained the sequence for the 88 amino acids of mature colicin Y101 peptide was amplified by PCR using plasmid pKY29 as a template and JMc12 and JMc27 as primers. The PCR product was cloned into the *Hin*dIII and *Kpn*I sites of pUC118, resulting in plasmid pDSY1. The fragment was sequenced in both directions to confirm that there were no errors compared with the nucleotide sequence of the structural gene for colicin Y101. Using *Hin*dIII and *Kpn*I the PCR product from pDSY1 was excised and cloned into these restriction sites in pRW19e (see Table 4.1). The resulting plasmid pDSY29 contained divergicin A signal peptide::colicin Y101 structural gene fusion with its transcription under the control of the

P32 promoter. As a result, the 3' end of the divergicin A signal peptide and structural and immunity genes were removed from pRW19e. Therefore, fusion of colicin Y101 structural gene to the divergicin A signal peptide was accomplished by replacing the divergicin A structural and immunity genes with colicin Y101 mature peptide gene in pRW19e. Lactic acid bacteria transformed with pDSY29 were tested for colicin production by the deferred inhibition test against *E. coli* DH5α and MH1. *E. coli* DH5α and MH1 containing pKY50 were used as resistant indicator and positive control for colicin production while lactic acid bacteria transformed with pMG36e and pRW19e were used as negative controls for colicin production.

4.3. Results

4.3.1. Comparison of inhibitory activity between colicin Y101 and colicin V

To compare the inhibition profiles of colicin Y101 and colicin V, similar sized fragments of pKY9 and pHK22 were used to construct colicin Y101 and colicin V recombinants using the same host strains and expression vectors (Fig. 4.1). For colicin V, approximately 7.2-kb *Hin*dIII-*Bgl*II fragment containing all four genes involved in colicin V production, immunity and export was excised from pHK22 and cloned into *Hin*dIII-*Bam*HI sites of pUC118 and pACYC184 resulting in plasmids pKV72 and pKV72-1, respectively. pHK22 is a derivative of pACYC184 that contains the 9.4-kb *Sal*I-*Hin*dIII fragment cloned from colicin V plasmid pColV-K30 (Gilson *et al.*, 1987). pKY9 is a derivative of pUC118 that contains a 6.6-kb *Hin*dIII fragment cloned from the native colicin Y101 plasmid that expressed colicin Y101 activity and immunity (Chapter 3). For colicin Y101, the 6.6-kb *Hin*dIII fragment containing all four genes involved in colicin Y production was excised from pKY9 and cloned into *Hin*dIII sites of

pACYC184 to create plasmid pKY9-1. Because of different restriction sites in pHK22 and pKY9, the 7.2-kb *Hin*dIII-*Bgl*II fragment (colicin V) and 6.6-kb *Hin*dIII fragment (colicin Y101) are the most similar DNA fragments to compare activity in this experiment. Both *E. coli* DH5α and MH1 were transformed with pKY9, pKY9-1, pKV72, and pKV72-1, respectively, yielding four different producers of each colicin. Colicin production was detected by deferred inhibition assay using non-transformed *E. coli* DH5α and MH1 as indicators.

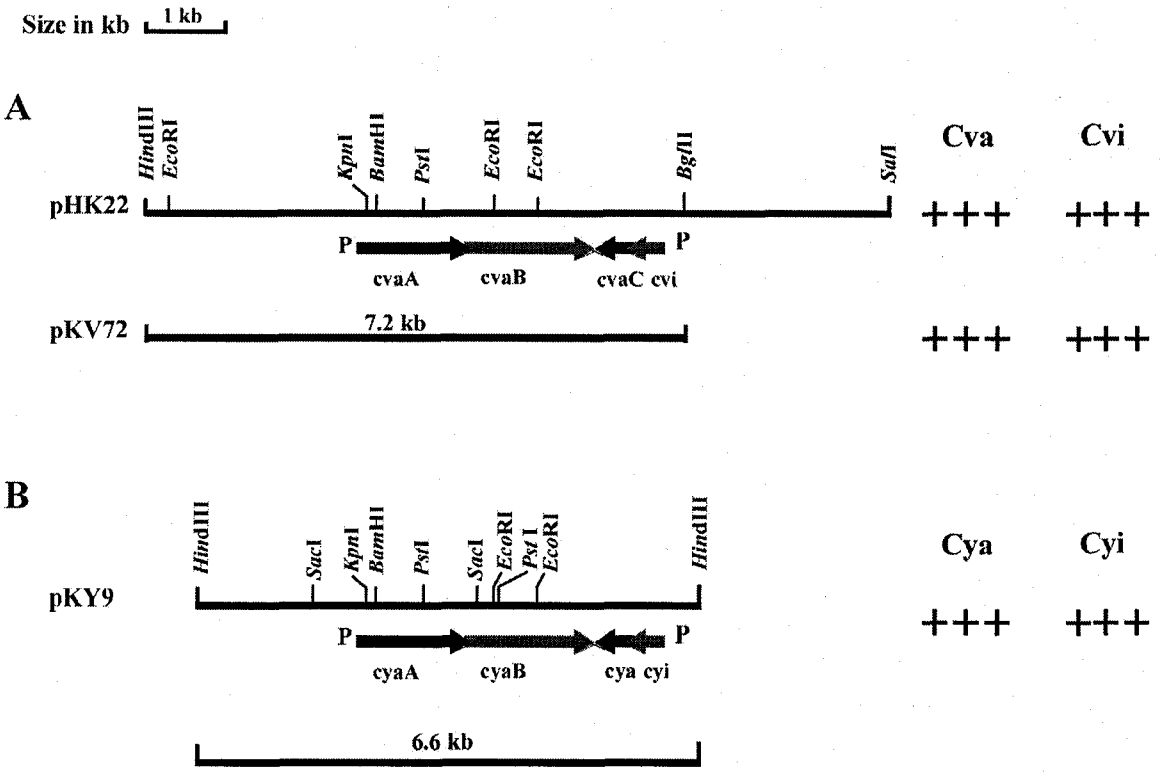

Figure 4.1 Restriction map of pHK22 and its derivative, pKV72 (A), and pKY9 (B). **(A)** pHK22 is a derivative of pACYC184 (adapted from Gilson *et al.*, 1987) and *Hin*dIII-*Bgl*II (7.2 kb) fragment was cloned into pUC118 to generate pKV72. **(B)** *Hin*dIII (6.6 kb) fragment from colicin Y101 native plasmid pKY1 was cloned into pUC118 resulting in pKY9 (Chapter 3)

As shown in Figure 4.2, the zones of inhibition against *E. coli* DH5α generated by DH5α containing either pKY9 or pKY9-1 were larger than those observed with DH5α containing pKY72 or pKY72-1. When *E. coli* MH1 was used as an indicator, no difference in size of the zones of inhibition was observed. Similarly, when *E. coli* MH1 was transformed with these four plasmids, the same results were obtained against both non-transformed *E. coli* DH5α and MH1 as indicator strains (data not shown). Larger zones of inhibition produced by colicin Y101 in most cases indicate that ColY101 may have a higher activity than ColV. On the other hand, the size of the zones of inhibition produced by both pUC118 derivatives, pKY9 (ColY101) and pKY72 (ColV) was much larger than those of pACYC184 derivatives (Fig. 4.2). This is because of the difference of copy number that the plasmid vector possessed.

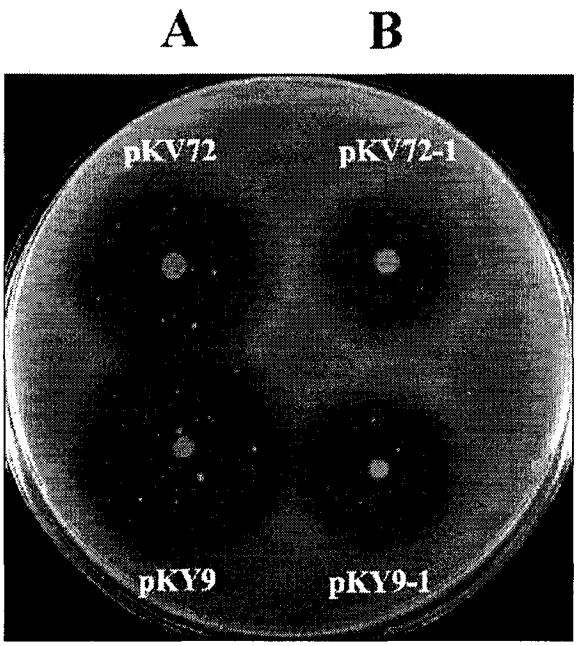

Figure 4.2 Deferred inhibition test against sensitive *E. coli* DH5α with colicin V (top) and colicin Y101 (bottom). *Hin*dIII-*Bg*lII (7.2 kb) fragment from pHK22 for colicin V and *Hin*dIII (6.6 kb) fragment from pKY1 for colicin Y101 were cloned into pUC118 (A) and pACYC184 (B), respectively.

Comparisons of the activity profiles of colicins V and Y101 determined by deferred inhibition test using *E. coli* DH5α containing each plasmid construct are shown in Table 4.2. The best results were observed from *E. coli* MH1 carrying pKY9. Some indicator strains that were sensitive to *E. coli* MH1 carrying pKY9 was not inhibited by *E. coli* MH1 carrying pKY9-1 and only the strains inhibited by colicin V were assessed in the activity spectrum of colicin Y101. This may be another evidence for a higher activity of ColY101 than ColV.

4.3.2. Deletion analysis in export genes

For CvaA$^-$ phenotype of colicin Y101, *Hin*dIII-*Kpn*I (4.24-kb), *Hin*dIII-*Bam*HI (4.1-kb), and *Hin*dIII-*Pst*I (3.52-kb) fragments were cloned from pKY50 into compatible restriction sites of pUC118 resulting in plasmids pKY50-KP, pKY50-BA, and pKY50-PS, respectively. Due to the absence of appropriate restriction sites in plasmid vectors pACYC184 and pMG36e, *Hin*dIII-*Bam*HI and *Hin*dIII-*Pst*I fragments were cloned into pACYC184 and pMG36e, respectively, resulting in plasmids pKY50-BA18 and pKY50-PS36 (Fig. 4.1). Although the size of the zones of the inhibition was noticeably reduced, very clear zones of inhibition were detected with transformants *E. coli* DH5α and MH1 carrying pKY50-KP, pKY50-BA and pKY50-PS, while no zones of inhibition were detected with transformants *E. coli* DH5α and MH1 carrying pKY50-BA18 and pKY50-PS36. Further deletions were made to construct CvaA$^-$B$^-$ phenotypes. The 2.66-kb and 2.0-kb *Hin*dIII-*Eco*RI fragments were excised from pKY50 and ligated into *Hin*dIII-*Eco*RI restriction sites of pUC118, yielding plasmids pKY50-EC26 and pKY50-EC20, respectively (Fig. 4.1). It was previously shown that pKY29 (CvaA$^-$B$^-$) produced active extracellular colicin Y101 (Chapter 3); however, *E. coli* DH5α and MH1 transformed with either pKY50-EC26 or pKY50-EC20 did not confer colicin Y101 activity to the

transformants. All of the clones described above were immune to colicin Y101 (Table 4.3).

Table 4.2 The comparison of inhibitory activity of colicin Y101 and colicin V against selective Gram-negative bacterial strains by the deferred inhibition assay.

Indicators	Colicin Y101[a]		Colicin V[b]	
	pKY9[c] (pUC118)	pKY9-1[c] (pACYC184)	pKV72[c] (pUC118)	pKV72-1[c] (pACYC184)
P. aeruginosa ATCC 15442	+++++	+++++	++++	++++
P. aeruginosa ATCC 27853	+++++	+++++	++++	++++
S. Choleraesuis ATCC 10708	+++	+	++	-
S. Choleraesuis ATCC 13312	+++	+	++	-
S. Enteritidis ATCC 13076	+++	F	+	-
S. Gaminara ATCC 8324	+++	+	++	F
S. Paratyphi ATCC 8759	+	+	-	-
S. Thompson ATCC 8391	F	-	-	-
S. Typhimurium ATCC 13311	+	F	+	F
S. Typhimurium ATCC 23564	+	+	F	-
S. Worthington ATCC 9607	F	-	-	-
Y. enterocolitica ATCC 23715	-	-	-	-
S. flexneri ATCC 12661	++	+	F	-
P. vulgaris ATCC 8427	-	-	-	-
P. agglomerans ATCC 27155	-	-	-	-
C. freundii ATCC 8090	-	-	-	-
C. intermedium ATCC 6750	+	F	-	-
K. ozaenae ATCC 11296	+	F	-	-
E. coli O157:H7 ATCC 12096	+++++	+++	++++	+++
E. coli O157:H7 ATCC 12902	+++++	+++	++++	+++
E. coli O157:H7 ATCC 13025	+++++	+++	++++	+++
E. coli O157:H7 ATCC 43895	+++++	+++	++++	+++

[a, b] For colicin Y101, *Hin*dIII (6.6 kb) fragment from pKY1 was cloned into pUC118 and pACYC184 resulting in pKY9 and pKY9-1, respectively. For colicin V, *Hin*dIII-*Bg*lII (7.2 kb) fragment from pHK22 was cloned into pUC118 and pACYC184 resulting in pKV72 and pKV72-1, respectively. +++++: >25 mm, ++++: 20-25 mm, +++: 15-20 mm, ++: 10-15 mm; +: <10 mm and clear, F: <10 mm and faint, -: no inhibition

[c] the host strain was *E. coli* DH5α

For colicin V, as shown in Figure 4.1, three fragments from pHK22 were cloned separately into pUC118 resulting in pKV-BK (4.14-kb *Bgl*II-*Kpn*I, CvaA$^-$), pKV-BB (4.0-kb *Bgl*II-*Bam*HI, CvaA$^-$) and pKV-BE (2.57-kb *Bgl*II-*Eco*RI, CvaA$^-$B$^-$). Both *E. coli* DH5α and MH1 were transformed with pKV-BK (CavA$^-$) or pKV-BB (CvaA$^-$) produced zones of inhibition against sensitive strains DH5α and MH1 even though the sizes of the zones of inhibition were dramatically reduced. However, cells that were transformed with pACYC184 containing the pKV-BB18 (4.0-kb *Bgl*II-*Bam*HI, CvaA$^-$) did not confer colicin V activity. The cells with a derivative of pUC118 containing *Bgl*II-*Eco*RI fragment (pKV-BE) did not produce colicin V. All of these colicin V phenotypes were immune to colicin V (Table 4.3).

Table 4.3 Colicin production by strains of phenotypes.

Colicins	Phenotypes	Vector	Plasmids[a]	Activity[b]	Immunity
Colicin Y101	**CyaAB**	pUC118	pKY50	35	Imm$^+$
	CyaA$^-$	pUC118	pKY50-KP	<8	Imm$^+$
		pUC118	pKY50-BA	<8	Imm$^+$
		pUC118	pKY50-PS	<8	Imm$^+$
		pACYC184	pKY50-BA18	0	Imm$^+$
		pMG36e	pKY50-PS36	0	Imm$^+$
	CyaA$^-$B$^-$	pUC118	pKY29	15	Imm$^+$
		pUC118	pKY50-EC26	0	Imm$^+$
		pUC118	pKY50-EC20	0	Imm$^+$
Colicin V	**CvaAB**	pACYC184	pHK22	25	Imm$^+$
	CvaA$^-$	pUC118	pKV-BK	<6	Imm$^+$
		pUC118	pKV-BB	<6	Imm$^+$
		pACYC184	pKV-BB18	0	Imm$^+$
	CvaA$^-$B$^-$	pUC118	pKV-BE	0	Imm$^+$

[a] In all cases, the host strain was *E. coli* DH5α

[b] Zones of inhibition were determined by the deferred inhibition assay. *E. coli* DH5α was used as indicator, the diameter (mm) of zone of the inhibition against indicator was determined.

4.3.3. Heterologous expression of colicin Y101 in lactic acid bacteria

Similar to colicin V production in lactic acid bacteria described by McCormick *et al.* (1999), heterologous expression of colicin Y101 was achieved in lactic acid bacteria using the signal peptide of divergicin A. The 15 amino acid N-terminal leader peptide (5'-MRTLTLNELDSVSGG-3') of colicin Y101 was replaced with the signal peptide (5'-MKKQILKGLVIVVCLSGATFFSTPQASA-3') of divergicin A (Fig 4.3). The DNA fragment encoding the mature peptide for colicin Y101 was amplified by PCR from pKY29 and cloned into the *Hin*dIII-*Kpn*I sites of pUC118 to create the plasmid pDSY1. A 295-bp *Hin*dIII-*Kpn*I fragment pDSY1 was cloned into the pRW19e resulting in pDSY29, replacing divergicin A structural gene and its immunity gene with colicin Y101 structural gene.

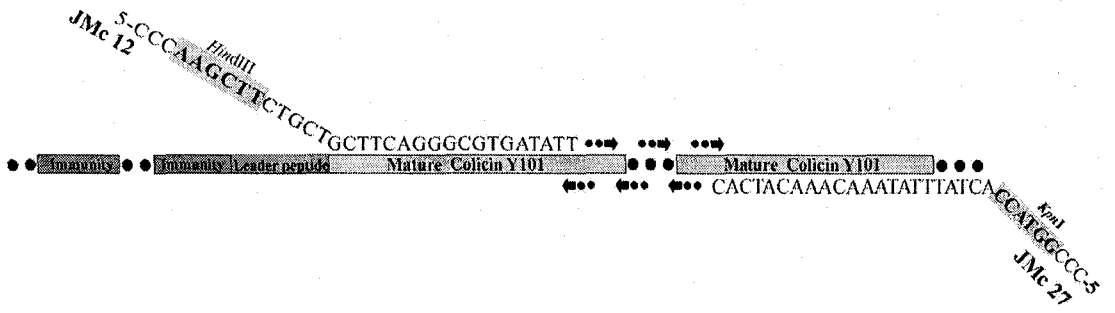

Figure 4.3 Schematic representation of PCR strategy to replace the double-glycine-type leader peptide of colicin Y101 with divergicin A signal peptide. The location and nucleotide sequence of two primers used for PCR and *Hin*dIII-*Kpn*I restriction sites are shown. The forward primer (JMc12) contains the C-terminus of divergicin A signal peptide (5'-CAAGCTTCTGCTG-3') and following 18 nucleotide of the N-terminus of colicin Y101 structure gene (GCTTCAGGGCGTGATATT). The reverse primer (JMc27) is complementary to the 3' end of the colicin Y101 structure gene next to *Kpn*I restriction site.

To compare the characteristics of colicin Y101 with those of colicin V, *C. maltaromaticum* UAL26, *C. maltaromaticum* LV17C, *C. divergens* LV13 and *Lactococcus lactis* IL1403 were used as host strains of pDSY29 (Table 4.1). Electrotransformation of plasmid pDSY29 into these four strains of lactic acid bacteria was successful.

All of these transformants produced similar zones of inhibition against sensitive *E. coli* DH5α and MH1 but they were not active against *E. coli* DH5α and MH1 containing pKY50 that encodes immunity for colicin Y101. Zones of inhibition produced by *E. coli* DH5α or MH1 containing pKY50 were noticeably larger than those produced by the four host strains of lactic acid bacteria containing pDSY29. Transformants containing pRW19e or pMG36e (data not shown) were used as negative controls and they were not active against *E. coli* DH5α and MH1 (Fig. 4.4).

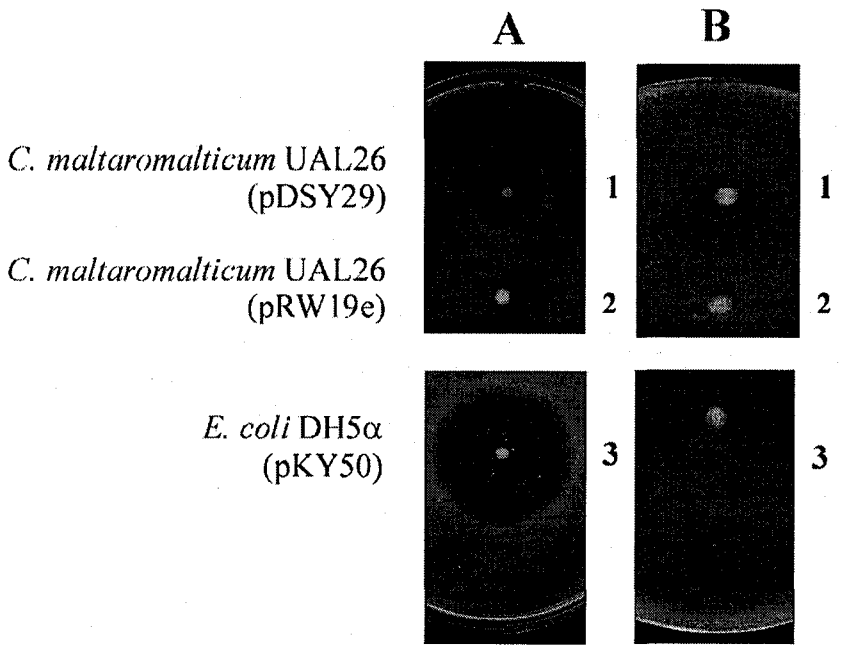

Figure 4.4 Deferred inhibition test with *C. maltaromaticum* UAL26 containing pDSY29 (1) and pRW19e (2) and *E. coli* DH5α containing pKY50 (3). Indicator strains were: (A) *E. coli* DH5α (sensitive to colicin Y101), and (B) *E. coli* DH5α carrying pKY50 (ColY101$^+$, Imm$^+$).

To localize colicin Y101 produced by heterologous host containing pDSY29, the culture supernatant from *C. maltaromaticum* UAL26 was collected, partially purified by XAD-8 column, concentrated, and separated by tricine-SDS-PAGE. Colicin Y101 produced by both homo- and heterologous expression migrated the same distance (Fig. 4.5). The molecular mass of the two samples determined by using MALDI-TOF mass spectrometric analysis ranged from 8,760 to 8,790 Da (data not shown). These results indicate that colicin Y101 could be exported by lactic acid bacteria using the signal peptide of divergicin A.

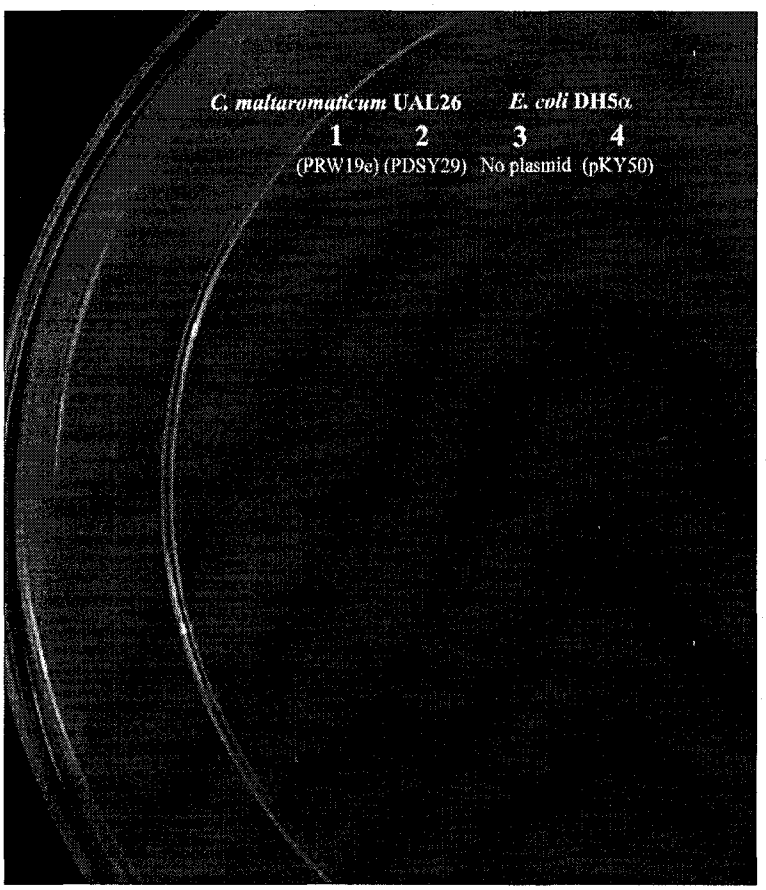

Figure 4.5 Localization of antagonistic activity by colicin Y101 produced by homo- and heterologous expression system in a tricine-SDS-PAGE gel. *E. coli* DH5α was used as the indicator strain in the overlay test. The culture supernatant from *C. maltaromaticum* UAL26 carrying pRW19e (lane 1, *dvn*$^+$, *dvi*$^+$) and pDSY29 (lane 2, cya$^+$) and *E. coli* DH5α (lane 3) and DH5α carrying pKY50 (lane 4, *cya*+, *cyi*+) were partially purified followed by concentration.

Similarly, a divergicin A signal peptide::colicin V fusion construct was created using pHK22 resulting in plasmid pDSV1 (same as pJKM37). This construct is the same derivative of pRW19e as pJKM37 described by McCormick *et al.* (1999). *C. maltaromaticum* UAL26 transformed separately with pDSV1 and pDSY29 were examined by deferred inhibition test against *Enterobacteriaceae* for comparison of their inhibitory profiles. Although zones of inhibition produced by both colicins from *C. maltaromaticum* UAL26 containing pDSY29 and pDSV1 were smaller than those of *E. coli* DH5α carrying pKY50 or pHK22, *C. maltaromaticum* UAL26 with pDSY29 or pDSV1 inhibited the growth of four test strains of *E. coli* O157:H7. However, the amount of colicins from *C. maltaromaticum* UAL26 containing pDSY29 was not sufficient to detect an inhibitory effect against other indicator strains that were previously shown to be sensitive to colicin Y101 or colicin V, including: *Salmonella, Shigella, Klebsiella* and *Citrobacter*. The same result was observed for *C. maltaromaticum* UAL26 containing pJKM37 (data not shown).

4.4. Discussion

A high incidence of colicinogenicity has been reported among strains of *E. coli*. Production of colicin gives a strain a competitive advantage, although the antibacterial activity of the colicins is often very narrow and it is generally limited to closely related strains. Bradley *et al.* (1991) evaluated the inhibitory activity of colicins against *E. coli* O157:H7 and showed that colicins G and H inhibited all 20 strains tested, while colicins E2 and V inhibited 12 and 18 of the strains, respectively. Murinda *et al.* (1996) also evaluated colicin activity against a set of 27 serotypically defined and genetically related diarrheagenic *E. coli* including serotypes O15:H-, O26:H-, O111:H-, and O157:H7. They showed that all 11 *E. coli* O157:H7 tested were sensitive to most colicins with mitomycin C induction and to colicins G and H without mitomycin C while colicin V inhibited only 2 out of 11 strains of *E. coli* O157:H7. Most other serotypes were sensitive to most colicins but not to colicins B, M, and V. Apart from that study (Bradley *et al.*, 1991), limited information is available on the inhibitory spectra of colicins against other strains of *Enterobacteriaceae*. Colicin Y101 is a natural variant of colicin V and the antibacterial effects of colicins V and Y101 on various strains of *Enterobacteriaceae* were evaluated (Chapters 2 and 3). From the study, colicins V and Y101 showed strong inhibitory activity against *E. coli* including serotype O157:H7 and they were active against strains of *Salmonella, Shigella, Klebsiella,* and *Citrobacter* spp. (mostly *Salmonella*). However, only the strains tested and sensitive to colicin V were included in the spectrum of colicin Y101. It was concluded that colicin Y101 had a higher specific activity than colicin V. To confirm this we cloned a 7.2 kb *Bgl*II-*Hin*dIII fragment of plasmid pHK22 containing all four genes required for colicin V production and an equivalent 6.6 kb *Hin*dIII fragment from native plasmid pKY1 of colicin Y101 into the same expression vector and introduced it into the same host. This indicated that colicin Y101 as a natural variant of

colicin V does not have a different antimicrobial inhibitory spectrum and that colicin Y101 might have a higher activity than colicin V.

Colicin Y101 is a natural variant of colicin V with two different amino acids in the mature peptide and an identical amino acid sequence in the N-terminal leader peptide (Chapter 3), the extracellular secretion of both colicins requires a set of dedicated export proteins, CvaAB/TolC. The CyaB protein for colicin Y101 differs from the CvaB protein for colicin V by four amino acids. CvaB is the ABC transporter located on the cytoplasmic membrane and has a typical cytoplasmic ATP-binding domain in its C-terminus. CvaB interacts with CvaA to facilitate efficient export of colicin V (CyaB for colicin Y101) and is unstable in the absence of CvaA (Gilson *et al.,* 1990). The periplasmic protein, CvaA, connects CvaB with TolC that is a chromosomally-encoded outer membrane protein. The leader peptides of colicins V and Y101 are removed by CvaB (CyaB for colicin Y101) to become the active form during the export process. Theoretically, colicins V and Y101 cannot be processed in the absence of CvaA or CvaB (CyaA or CyaB for colicin Y101, respectively). However, we observed clear zones of inhibition produced by both $CyaA^-$ and $CyaA^-B^-$ phenotypes of colicin Y101 (Chapter 3). In the earlier studies of colicin V, Frick *et al.* (1981) and Gilson *et al.* (1987, 1990) obtained decreased levels of internally active colicin V from the $CvaA^-$ and $CvaB^-$ mutants.

Hence, it was of interest to examine externally active colicins V and Y101 in $CvaA^-B^-$ ($CyaA^-B^-$) phenotypes. In this study, we carried out a number of experiments to clarify these unusual results. $CyaA^-$ and $CyaA^-B^-$ phenotypes of colicin Y101 were constructed with deleted fragments within the region of CyaAB operon into pUC118, pACYC184, and pMG36e. pUC118 and pACYC184 derivatives for colicin V phenotypes were also prepared for comparison (Fig. 4.1). $CyaA^-$ phenotypes of colicin Y101 produced clear zones of inhibition when a construct with deleted fragments in the *cyaA*

gene was cloned into pUC118 but not in pACYC184 nor pMG36e. The same results were observed from CvaA$^-$ phenotypes of colicin V. This is understandable if CyaB (CvaB for colicin V) is translated because both colicins Y101 and V could be processed to remove their N-terminal leader peptides by CyaB (CvaB) that acts as the leader peptidase and helps these colicins to cross the inner membrane. On the other hand, no detectable zones of activity were observed from CvaA$^-$B$^-$ mutants of colicin V or CyaA$^-$ B$^-$ mutants of colicin Y101 using the different plasmid vectors except with pKY29. Therefore, it was concluded that active colicin Y101 produced by CyaA$^-$ mutants was dependent upon the vector, suggesting over-expression of colicin Y101, accumulation of the colicin in the periplasmic space and leakage from the cell. However, translation of CyaB is unlikely because it is co-translated with CyaA. Alternately, as suggested in Chapter 3, two possible in-frame ORFs that are located immediately downstream of CyaB could explain this phenomenon (for detailed discussion see Chapter 3). This speculation was supported by the results obtained with these CyaA$^-$B$^-$ mutants in pKY29, pKY26 and pKY20. The cells carrying pKY29 might produce truncated inframe CyaB by *orf*2, but the cells carrying either pKY26 or pKY20 did not. However, the possibility of two translation products by *orf*1 and *orf*2 still need to be investigated.

Colicin V shares many features with class IIa peptide bacteriocins produced by Gram-positive lactic acid bacteria (Håvarstein *et al.,* 1994, 1995). The double glycine leader peptide is removed by CvaB that is similar to other ABC proteins that are involved in bacteriocin export. Production of colicin V and other bacteriocins using heterologous ABC transporters in other species has been reported (Van Belkum *et al*, 1997). Zhang *et al.* (1995) showed that *omp*A signal peptide fused to mature structural peptide for colicin V was transported into the periplasmic space by the *sec*-pathway. In addition, McCormick *et al.* (1999) demonstrated the extracellular secretion of colicin V using the signal peptide of divergicin A and the *sec*-pathway in lactic acid bacteria.

In this study, we carried out several related experiments to characterize the colicin Y101 system and its heterologous expression using the *sec*-pathway in lactic acid bacteria. We also accomplished the production of colicin Y101 by the *sec*-pathway in lactic acid bacteria also using the divergicin A signal peptide and compared inhibitory spectrum of colicins V and Y101 against strains that were previously shown to be sensitive. Our results showed that both colicins V and Y101 inhibited all of the strains of *E. coli* that were tested including strains of *E. coli* O157:H7. However, neither of these colicins inhibited the growth of other strains of *Enterobacteriaceae* that were previously shown to be sensitive. This may be due to low production of the colicins by the lactic acid bacteria.

Recent research has focused on developing heterologous expression systems in lactic acid bacteria that allow production of multiple bacteriocins and therefore offers a tool for enhanced effectiveness against target organisms. We have shown that colicin Y101 from *E. coli* can be produced in lactic acid bacteria by the general secretion pathway and at concentrations that efficiently inhibit *E. coli* O157:H7. These results have interesting possibilities for further development and extension of the application of bacteriocins in food preservation. It seems that as a general rule bacteriocins produced by Gram-positive bacteria are not active against Gram-negative bacteria, hence strategies using colicin-producing lactic acid bacteria that produce defined bacteriocins of interest could be a distinct advantage for application in the food industry.

4. 5. References

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Our research group has focused on developing biopreservation systems using bacteriocins of lactic acid bacteria. Bacteriocins are ribosomally synthesized antibacterial metabolites that are produced by bacteria with inhibitory activity generally against closely-related strains or species. In the past decade, application of bacteriocins as food biopreservatives has received considerable attention because of their ability to prevent growth of foodborne pathogens such as *Listeria* and *Clostridium* species. For example, nisin is a bacteriocin produced by some strains of *Lactococcus lactis* subsp. *lactis* that is active against a broad range of Gram-positive bacteria. Nisin has been approved as a food preservative in numerous countries (Delves-Broughton, 1990). Pediocin PA-1/AcH is another bacteriocin of lactic acid bacteria, for which several U.S. and European patents have been issued but it is not yet in commercial use (Stiles, 1996). A limitation of the use of bacteriocins from lactic acid bacteria against foodborne pathogens is the fact that Gram-negative pathogens such as *E. coli* O157:H7, *Salmonella, Shigella, Yersinia* spp. and other *Enterobacteriaceae* are not inhibited by bacteriocins from Gram-positive bacteria, including nisin and pediocin PA-1/AcH, without an additional sublethal stress (Cutter and Siragusa, 1995; Stevens *et al.*, 1991, 1992).

Bacteriocins such as colicins and microcins produced by Gram-negative bacteria differ from those produced by lactic acid bacteria. Colicins are specifically active against *E. coli* and other closely related strains of *Enterobacteriaceae* (Pugsley and Oudega, 1987). Several studies indicated that colicinogeny is more common among pathogenic than commensal isolates of *E. coli* and may be more prevalent among human isolates than animal isolates (Hardy, 1974; Selander *et al.*, 1987). The incidence of colicinogeny

among strains of *E. coli* O157:H7 was reported to be between 33 and 50% and the almost universal production of colicin D was established (Frost *et al.*, 1989; Scotland *et al.*, 1987; Whittam *et al.*, 1993).

To date, more than 25 different colicins have been identified. The frequency of different types of colicins varies substantially between populations. Colicin-producing strains generally synthesize an immunity protein that provides protection against their own colicin. On the other hand, the susceptibility of target cells to colicins depends on adsorption rate of the specific surface receptor and immunity specificity resulting in a very narrow target range within *E. coli* and its close relatives. Levels of colicin resistance among other enteric bacteria are as high or higher than levels detected within *E. coli* due to trends in intraspecific action of colicin (Riley and Gordon, 1999). No clear description of a co-relationship between immunity and other colicins has been established but the fact that producer cells are protected against their cognate colicin is clear.

The inhibitory activity of colicins against strains of *E. coli* serotype O157:H7 has been reported. Bradley *et al.* (1991) established that colicins G and H inhibited all 20 strains of *E. coli* serotype O157:H7 that they tested; while colicins E2 and V inhibited 12 and 18 of the strains, respectively. According to Bradley *et al.* (1991), colicins A, B, K, D and Ia had no effect against these strains of *E. coli* O157:H7. Murinda *et al.* (1996) also evaluated the sensitivity of a set of 27 serotypically defined and genetically related diarrheagenic strains of *E. coli* (DEC) to several colicins. All of the strains of *E. coli* O157:H7 tested were inhibited by colicins E1 to E8, G and H. Serotypes *E. coli* O26:H11 and O26:H- were shown to be the most sensitive to all of the colicins tested, while *E. coli* serotype O111:H- was the least sensitive. However, only 2 out of 11 strains of *E. coli* O157:H7 were inhibited by colicin V. This report indicated that colicins with strong effectiveness against strains of *E. coli* O157:H7 may have the potential for application in the control of this undesirable organism. It is, therefore, possible that purified colicin

may have potential for application as biopreservatives for control of *E. coli*. Alternatively, well-screened food grade microorganisms such as lactic acid bacteria could be the host strains for production of colicin(s) and then directly applied to foods.

The primary purpose of this thesis was to isolate and identify a novel colicin from *E. coli*. The overall goal was the development of a powerful bacteriocin expression system that could produce multiple bacteriocins active against Gram-negative and Gram-positive bacteria using food grade lactic acid bacteria that consequently could kill undesirable Gram-negative and Gram-positive bacteria. Initially, we attempted to determine the frequency of colicinogeny in *E. coli* isolated from meat. We discovered that about 20% of the meat isolates were colicinogenic. We found one particularly interesting colicin-producing strain, *E. coli* KB101. Its colicin displayed atypical characteristics, including the fact that its production was not affected by mitomycin C, indicating that it is non-SOS inducible, which is a common feature of most colicins.

Because receptor specificity and specific immunity of colicins are considered to be key factors for the killing action of colicins (Konisky, 1982; Pugsley and Oudega, 1987), the homologous immunity protein does not confer immunity to other types of colicin. The antibacterial compound of strain *E. coli* KB101 (denoted colicin Y101) is of special interest and was chosen for further study because of its atypically small size (8 kDa) and its relatively broad activity spectrum against a large number of *Enterobacteriaceae,* including *E. coli* O157:H7, *Salmonella, Shigella* and *Klebsiella* spp. It was also active against strains of *Pseudomonas aeruginosa* and *Citrobacter intermedium*. Colicin Y101 was shown not to be 'SOS' inducible and it did not require a lysis protein for its release, as is the case for most other colicins (Gilson *et al.,* 1990). Colicin Y101 is proteinaceous and insensitive to lipase, catalase and α-amylase. It is stable at temperatures from 70°C to boiling and over a wide pH range from pH 3 to 10. With these advantageous characteristics, colicin Y101 has the potential for application as

an effective method for controlling *E. coli* O157:H7 and other *Enterobacteriaceae* in food preservation and processing.

Colicin Y101 was shown to be plasmid encoded. Strain KB101 has three plasmids, pKY1, pKY2, and pKY3, all of which were transferred to *E. coli* strains DH5α and MH1. Transformants containing pKY1 were colicin-positive and immune to *E. coli* KB101, while transformants with pKY2 or pKY3 were sensitive to *E. coli* KB101 and they were ColY101-negative. To characterize and distinguish this colicin from other known colicins, purification was successfully achieved from the supernatant of the transformant *E. coli* KY1 by hydrophobic interaction chromatography, cation exchange chromatography and reverse phase HPLC. The molecular weight (MW) of colicin Y101 was estimated by tricine-SDS-PAGE to be 8.1 kDa, similar to colicin V (8735.8 Da) and a molecular weight of 8,765.8 Da was confirmed by MALDI-TOF mass spectrometric analysis.

The genetic determinants of colicin Y101 were identified from a fragment of the 120-kb native plasmid pKY1. Restriction analysis on this plasmid revealed the location of the genetic determinant for colicin Y101 production, immunity and export on a 5.0-kb *Hin*dIII-*Sac*I fragment of pKY1. The four ORFs for ColY101 were organized in two converging operons that showed extensive homology with the cloned region of pColV-B188 and pColV-K30 for colicin V (Frick *et al.*, 1981; Gilson *et al.*, 1987, 1990). A search for amino acid homology revealed extensive identity between colicin Y101 and colicin V polypeptides. The two genes involved in colicin Y101 export had 100% identity to *cvaA* and 99.4% identity to *cvaB*. Colicin Y101 structural and immunity genes showed 98% and 97% identity to colicin V structural and immunity genes, respectively. Therefore, it may be assumed that ColV and ColY101 represent a divergent genetic evolution that probably occurred by natural mutagenesis.

Gilson *et al.* (1990) reported that the structural gene (*cvaC*) for colV encodes

103 amino acid residues and that the secreted translation product of *cvaC* is processed to remove its N-terminal leader peptide containing a specific Gly-Gly cleavage site. This so-called double-glycine-type leader peptide is common for class IIa bacteriocins of lactic acid bacteria and it is associated with a dedicated bacteriocin export system (Fath *et al.,* 1994; Klaenhammer, 1993; Nes *et al.,* 1996). Bacteriocins in this group are produced as precursors with a N-terminal leader peptide of 18 to 24 amino acids (Håvarstein *et al.*, 1994; Klaenhammer, 1993; Van Belkum *et al.*, 1997). These dedicated export systems require at least two membrane-bound proteins: a transporter protein of the **A**TP **B**inding **C**assette (ABC) superfamily and an accessory protein (Franke *et al.*, 1996; Gilson *et al.*, 1990; Higgins, 1992; Stoddard *et al.*, 1992; Van Belkum and Stiles, 1995). The ABC transporters are responsible for cleavage of the N-terminal leader peptide and they are involved in transportation of the mature peptide out of the cell. The function of the accessory protein remains unclear in bacteriocins from Gram-positive bacteria (Fath and Kolter, 1993; Håvarstein *et al.*, 1995). The dedicated protein export system has also been shown to be involved in the secretion of *E. coli* α-hemolysin (HlyBD) and *Erwinia* protease (PrtDEF). Both proteins of the ABC superfamily (CvaA, HlyB, and PrtD) and an accessory protein referred as the MFP family (CvaB, HlyD, and PrtE) in Gram-negative bacteria are structurally and functionally related. In Gram-negative bacteria, an outer membrane protein such as TolC in colicin V and α-hemolysin export and PrtF in *Erwinia* protease export is required for the extracellular secretion of protein or peptide (Fath and Kolter, 1993; Skvirsky *et al.*, 1995). The export of colicin V was shown to be mediated by substitution with HlyBD and PrtDEF proteins (Fath *et al*, 1991).

Similar to colicin V, the structural and immunity genes of colicin Y101 are co-transcribed in the same operon. The initiation codon of colicin Y101 structural gene (*cya*) overlaps with the end of the immunity gene (*cyi*). The 15-amino-acid double-glycine-type leader peptide of colicin Y101 is identical to the leader peptide of colicin V.

Differences between colicin Y101 and colicin V involved replacements of two amino acids in positions 49 (Ile^{49} to Val) and 86 (Ala^{86} to Asp). The calculated molecular mass of colicin Y101 mature peptide is 8765.8 Da. The immunity protein of colicin Y101 consists of 78 amino acids. Two differences in amino acid composition were found in positions 9 (Glu^{9}→Asp) and 23 (Ile^{23}→Met) compared with the colicin V immunity protein. These two amino acid replacements did not confer changes in immunity; however, the colicin V producer carrying plasmid pHK22 (Gilson *et al.*, 1990) was not fully immune to the colicin Y101 producer carrying plasmid pKY50. This might be due to the over production of ColY101 by pKY50, which is a pUC118 derivative containing the 5.0-kb ColY101 gene cluster, *cyaA, cyaB, cyaC, cyi*, (Chapter 3). According to Gilson *et al.* (1990) and Fath *et al.* (1992), the immunity protein (Cvi) of colicin V spans the inner membrane, localizing its N- and C-termini in the cytoplasmic space.

The CvaAB/TolC dedicated export protein complex is essential for secretion of colicin V (Fath *et al.,* 1992, Fath and Kolter, 1993; Gilson *et al.,* 1990; Skvirsky, 1995). From the sequence analysis, two ORFs related to export genes of colicin Y101 showed 100% identity to CvaA and 99.4% identity to CvaB. Four amino acid differences were found in CyaB of colicin Y101 in positions 305 (Cys→305Phe), 486 (Asp→486His), 496 (Gly→496Asp), and 566 (His→566Gln). These two export genes overlap the region between *cyaA* terminating and *cyaB* initiation codons. CvaA (CyaA for colicin Y101) consists of 413 amino acids while CvaB (CyaB for colicin Y101) consists of 698 amino acids. The ABC transporter, CvaB (CyaB for colicin Y101) has six potential transmembrane domains located in the inner membrane with a typical ATP-binding domain in a C-terminal cytoplasmic region (Fath *et al.,* 1992). CvaB is believed to interact with CvaA and its translation level may be reduced with the absence of CvaA translation by preventing ribosome loading because of co-translation with CvaA (Hwang *et al.*, 1997). Most importantly, in colicin V export, CvaB has been shown to provide

leader peptidase activity (Fath *et al.*, 1994; Håvarstein *et al.*, 1995; Venema *et al.*, 1995). Although the role of CvaA is unclear in export, it appears that CvaA functions to form a bridge that connects the ABC protein to the outer membrane protein TolC to connect the inner and the outer membranes (Fath *et al.*, 1994; Hwang *et al.*, 1997).

Based on facts known for colicin V, colicin Y101 should not be properly processed in the absence of CyaA or CyaB. However, clear zones of inhibition were observed for both CyaA$^-$ and CyaA$^-$B$^-$ phenotypes of colicin Y101 producers (Chapter 3). In an earlier study of colicin V, Gilson *et al.* (1987, 1990) and Frick *et al.* (1981) reported a decreased level of internally active colicin V from mutants of CvaA and CvaB, but they did not observe active colicin V in the extracellular medium from these mutants. Our results confirmed that active colicin Y101 produced by CyaA$^-$ phenotypes was dependent upon the expression vector. We postulated that colicin Y101 precursor from CyaA$^-$ phenotypes could be processed to remove its N-terminal leader peptide by the leader peptidase activity of CyaB. Then mature colicin Y101 could be over expressed and accumulated in the periplasmic space causing damage in the outer membrane and leakage out of the cells. On the other hand, we could also find active transformants of the CyaA$^-$ B$^-$ phenotype carrying plasmid pKY29. Interestingly, two possible in-frame ORFs that were located immediately downstream of CyaB were found in plasmid pKY29. The two possible promoter sequences of –35 and –10 and probable ribosomal binding sites (RBS) were identified (see chapter 4 for details). The *orf1* could encode an in-frame protein of 621 amino acids while *orf*2 could encode an in-frame protein of 566 amino acids. These two putative *orf*s appeared to encode a truncated form of CyaB with the ATP-binding cassette (ABC) domain in its C-terminus. Two truncated in-frame proteins in CyaB is translated independently and their N-termini could function to cleave the leader of colicin Y101 pre-peptide and thus active colicin Y101 could cross the inner membrane. This speculation might be supported by the observations from three CyaA$^-$B$^-$ phenotypes,

pKY29, pKY26 and pKY20. The cells carrying pKY29 were colicin Y101 positive and might encode deleted CyaB by *orf*2, but the cells carrying pKY26 and pKY20 did not. Apparently CyaA and CyaB are necessary for the proper secretion of colicin Y101 and CyaB could play a key role as a leader peptidase for export.

Inhibitory profiles of colicin Y101 and colicin V were compared using the same host strains and expression vectors. Because of minor differences in restriction sites in plasmids pKY9 (colicin Y101) and pHK22 (colicin V), a similar DNA fragment (colicin V: 7.2-kb *Hin*dIII-*Bgl*II; colicin Y101: 6.6-kb *Hin*dIII) containing all four genes involved in production, export, and immunity were cloned and ligated with designated vectors to construct colicin Y101 and V recombinants. Although the difference in the antibacterial spectra of colicin Y101 and ColV was not great, the inhibitory profile of colicin V is apparently included in that of colicin Y101. Moreover, the zones of inhibition generated by colicin Y101 were larger than those produced by colicin V, indicating that colicin Y101 may be a better option for application in food. The same results were observed for other host strains of *E. coli* and expression vectors.

Previously, colicin V was produced by lactic acid bacteria by replacing the 15 amino acid N-terminal leader peptide of colicin V with the signal peptide of divergicin A (McCormick *et al.*, 1999). Heterologous expression of colicin Y101 using signal peptide of divergicin A in lactic acid bacteria was also achieved in this study. Divergicin A is a bacteriocin of *Carnobacterium divergens* LV13 that is produced as a prepeptide and it is exported from the cell by the general protein secretion (*sec*) pathway. The prepeptide consists of a classical N-terminal signal peptide of 29 amino acids and a mature peptide of 46 amino acids (Van Belkum *et al.*, 1997; Worobo *et al.*, 1995). Using the heterologous expression system, we confirmed production of colicins Y101 and V by heterologous lactic acid bacteria. However, the comparison of their killing effect was not possible because of low production. Neither colicin Y101 nor V inhibited the growth of

Enterobacteriaceae that were shown to be sensitive to these colicins produced by their own secretion method in an *E. coli* host. However, all strains of *E. coli* O157:H7 tested were effectively inhibited by colicin Y101 and colicin V produced by LAB.

There has been interest in the potential to apply bacteriocins including colicins in food systems. Researchers in our laboratory have constructed multiple bacteriocin expression systems for lactic acid bacteria that have the potential to offer an excellent tool for enhanced overall effectiveness against target organisms. It is noteworthy that expression of bacteriocins with a double glycine leader peptide can possibly be achieved by a heterologous system in a heterologous host (Allison *et al.,* 1995; McCormick *et al.,* 1996; Van Belkum *et al.,* 1997). For example, colicin V could be secreted in *L. lactis* via the lactococcin A dedicated export machinery. In addition, divergicin A under the control of the *sec*-dependent general secretion pathway could be secreted by substitution of the leader peptides of either leucocin A, lactococcin A or colicin V. On the other hand, it was also shown that the *sec*-dependent secretion system also allows secretion of the double glycine leader dependent bacteriocins by replacing their leaders with a signal peptide. The signal peptide of divergicin A can direct the secretion of carnobacteriocin B2 (McCormick *et al.*, 1996) and enterocin B (Franz *et al.*, 1997) that are generally exported by a dedicated export system. Replacement of the colicin V leader peptide with a signal peptide of divergicin A could also direct colicin V export in lactic acid bacteria (McCormick *et al.*, 1999). We also accomplished colicin Y101 production by *sec*-pathway in lactic acid bacteria through divergicin A signal peptide (Chapter 4). Strategies for development of colicin-producing lactic acid bacteria would enable the inhibitory spectrum of lactic acid bacteria to be extended to Gram-positive and Gram-negative organisms.

In summary, the overall objective of this thesis was partially fulfilled. A colicin-producing *E. coli* KB101 was isolated from ground beef and determined the

characteristics of its colicin. Colicin Y101 is a non-SOS-inducible proteinaceous antibacterial substance. Colicin Y101 exhibited a strong inhibitory effect against the growth of strains of *E. coli* including serotype O157:H7 and *Salmonella* and it was also active against strains of *Shigella, Klebsiella* and *Citrobacter* spp. The bacteriocin is relatively stable to heat treatment and over a wide range of pH. These facts suggest that it would be a good candidate for use in foods. Unlike most other known colicins, it has a small molecular size of 8,776.6 Da. The genetic determinants for its production, immunity and export were identified and nucleotide analysis revealed that colicin Y101 was a natural variant of colicin V with differences in two amino acids. Its leader peptide was identical to that of colicin V.

Colicin Y101 from *E. coli* KB101 could be produced in lactic acid bacteria via the general secretion pathway. In this case, the host lactic acid bacteria do not need to produce immunity protein because colicins from *E. coli* are not active against Gram-positive bacteria. Even though the secretion level by the heterologous system and host was decreased, strains of *E. coli* O157:H7 were efficiently inhibited. These results raise the interesting possibility of further development and extension of potential bacteriocin applications in foods. Because bacteriocins from Gram-positive bacteria do not have antibacterial activity against Gram-negative bacteria and the most common causes of foodborne illness are Gram-negative bacteria, strategies using colicin-producing lactic acid bacteria that produce defined bacteriocins of interest will be the great advantage to ensure the food safety.

5. 1. References.

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