MOLECULAR ANALYSIS OF MICROCIN 24: GENETICS, SECRETION AND MODE OF ACTION OF A NOVEL MICROCIN.

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Biology in the University of Canterbury.

GRAHAM JAMES O'BRIEN

1996

DEDICATION.

To Lee-ann.

Romans16: 27.

"to the only wise God be glory for ever through Jesus Christ! Amen."

ACKNOWLEDGMENTS.

I would like to thank Assoc. Prof. H.Khris Mahanty for his supervision of this project and his many ideas and enthusiasm which has guided this work. To both Khris and Abanti, many thanks for their hospitality and friendship.

Many thanks to Beth Robson and Sam Court for their technical assistance during this research and to my associate supervisor Dr. J.A Heinemann for his comments on this work. Thanks also to Dr. F.Y.T Sin, Dr. M.H. Turnbull and Dr. J.D. Klena for reading the draft. A note of special appreciation goes to all the students in the Molecular Genetics Laboratory both past and present who have made my time there a great experience.

My thanks to the staff of the Plant and Microbial Sciences Department who have been willing to help with equipment and other cries for help and especially Dougal Holmes for his photographic expertise.

I would like to acknowledge the assistance of Dr. M. Kennedy with the DNA sequencing, Dr. A.P. Pugsley for supplying the colicin reference, Dr. I.L. Lamont for supplying the Fur strains and Prof. R.E. Wooley for performing the embryo lethality assay. Many thanks to Dr. S.T. Chambers and Dr. B. Peddie from Christchurch Hospital for their initial and continued interest in this project.

The financial assistance provided by the University of Canterbury through the Doctoral Scholarship scheme and the Health Research Council of New Zealand who awarded me a Postgraduate Scholarship (94/142) for the duration of this work is gratefully acknowledged.

Thanks to my parents Dennis and Margaret, for their support and encouragement over the years and especially for the use of their computer. Thanks also to Mr D.M. Petrie for proof reading parts of this work.

Lastly, I would like to thank my wife Lee-ann who has supported me through the good and the bad. I could not have done this without her love and understanding. We can now look forward to the new life ahead of us.

ABSTRACT.

Colicins and microcins are proteinaceous antimicrobial agents produced by members of the *Enterobacteriaceae* which are active against other members of this family. Colicin 24 is a novel bacteriocin produced by a uropathogenic strain of *Escherichia coli* isolated at Christchurch Hospital. Through detailed genetic analysis of the DNA encoding this toxin and assaying the toxic activity, colicin 24 was re-classified as microcin 24 and has been shown to have a similar genetic organisation to that of colicin V and a novel mode of activity.

The region of DNA encoding microcin 24 was subcloned from pGOB34 into pBR322 generating pGOB18 (5.44kb). Mutagenesis, DNA sequencing and trans-complementation identified two regions with high sequence similarity and functional homology to the ColV transporters CvaA and CvaB. The insert DNA of pGOB18 was sequenced in both directions and has been found to contain 5267bp encoding five open reading frames, *mdbA*, *mtfI*, *mtfS*, *mtfA* and *mtfB*, forming three operons *mdbA*, *mtfI/mtfS* and *mtfA/mtfB* all of which were transcribed in the same direction. The predicted protein products of all the open reading frames except *mtfB* were confirmed by expressing the genes in minicells. Further mutagenesis and trans-complementation has identified *mdbA* as a *cis* acting positive regulatory gene with sequence similarity to the histone-like proteins. The *mtfI* and *mtfS* genes were confirmed as the Mcc24 immunity gene and the Mcc24 structural gene respectively. The genes *mtfA* and *mtfB* were found to encode the transport proteins homologous to CvaA and CvaB respectively, with *mtfB* encoding a protein which is a member of the ABC family of bacterial transporters. Transport also requires the TolC outer membrane protein. Analysis of the *mtfS* DNA sequence has identified a double glycine leader sequence, making MtfS the second microcin after ColV to belong to this class of peptide antibiotics. Experimental evidence suggested that unlike ColV, Mcc24 is inactive within the producing cell, however both toxins require the ABC transporter for post-translational modification of the pre-peptide.

The regulation of Mcc24 synthesis is controlled by the interaction between σ^S, Fur, and MdbA, encoded by the *mdbA* gene. Analysis of the promoter sequences has identified putative regions of DNA bending which might facilitate the binding of σ^S and

MdbA. A Fur-box with good sequence similarity to the consensus Fur-box has been identified in the *mtfI/mtfS* promoter and is the proposed site for Fur binding.

The activity spectrum of Mcc24 is restricted to enteric bacteria and SemA, the MccE492 receptor, is also required as the receptor for Mcc24. Extracts of Mcc24 have been found to degrade both linearised and covalently closed circular DNA *in vitro*. The activity is absent in extracts from *mtfS*⁻ strains, suggesting that Mcc24 inhibits the growth of sensitive cells by degrading DNA. The effect of Mcc24 expression on the virulence of *E. coli* was tested using the embryo lethality assay, however unlike ColV which increases the virulence of strains, the expression of Mcc24 did not appear to have a significant effect on *E. coli* virulence in this system.

TABLE OF CONTENTS.

CHAPTER (Continued). **PAGE**

LIST OF ABBREVIATIONS.

$A_{600/550/420}$	absorbance at 600, 550 or 420 nanometers
AAA-met/cys	all amino acids except methionine and cysteine
Amp^R/Amp^S	resistant/sensitive to ampicillin
Bis	N,N'-methylene-bis-acrylamide
BLAST	basic local alignment search tool
bp	base pair
cfu	colony forming units
CIP	calf intestinal alkaline phosphatase
Col	colicin
ColV	colicin V
$ColV^+/ColV^-$	produces/does not produce colicin V
$ColV^R$	resistant to colicin V
cpm	counts per minute
EDTA	ethylenediaminetetra-acetic acid
IHF	integration host factor
Imm^+/Imm^-	immune/not immune to microcin 24
Kan^R/Kan^S	resistant/sensitive to kanamycin
kb	kilobases
kDa	kilodaltons
mA	milliamps
Mcc	microcin
Mcc^+/Mcc^-	produces/does not produce microcin
Mcc^R/Mcc^S	microcin resistant/microcin sensitive
$Mcc24^+/Mcc24^-$	produces/does not produce microcin 24
$Mcc24^N$	produces a normal level of microcin 24
$Mcc24^{IN}$	produces an increased amount of microcin 24
$Mcc24^{RED}$	produces a reduced amount of microcin 24
$Mcc24^R$	resistant to microcin 24
min	minute

ABBREVIATIONS (Continued).

m.o.i	multiplicity of infection
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pfu	plaque forming units
PEG	polyethylene glycol
rpm	revolutions per minute
sec	seconds
SDS	sodium dodecyl sulphate
$Strep^R/Strep^S$	resistant/sensitive to streptomycin
Suc^R/Suc^S	resistant /sensitive to sucrose
TEMED	N,N,N',N'-Tetra-methylethylenediamine
Tet^R/Tet^S	resistant/sensitive to tetracycline
w/v	weight per volume

LIST OF FIGURES.

FIGURE (Continued). **PAGE**

FIGURE (Continued). **PAGE**

LIST OF TABLES.

CHAPTER 1.

INTRODUCTION.

The ability of one organism to inhibit the growth of another by the production of an antibiotic substance is a phenomenon seen widely among prokaryotic species. The term antibiotic refers to substances produced by one organism which are harmful to other organisms (Davies, 1990). Hopwood (1978) defined "true" antibiotics as small, non-typical compounds which are active against a wide range of organisms. These inhibitory substances are secondary metabolites produced by multi-enzyme processes, after the cessation of cellular growth (Kleinkauf and van Döhren 1987). Antibiotics are thought to play a natural role in biological interactions, where one microorganism inhibits the growth of other microorganisms in nutrient limiting environments (Martin and Demain, 1980; Hansen, 1993). Davies (1990) has argued that the antibiotic property of these substances might result from an original function as effectors or cofactors in the primeval translation machinery. It is proposed that secondary metabolites are primitive molecules, exerting their antibiotic activity through interactions with conserved sites on cellular macromolecules, for example ribosomes.

Bacteriocins are a class of antibiotic which differ from the classical antibiotics in that they are proteinaceous and are active against closely related species. Bacteriocins can be defined as bacterial peptide or protein antibiotics which are ribosomally made or derived from gene-encoded precursor peptides and toward which the producing strain possesses a self-protection mechanism (Sahl, 1994; Jack, 1995). Hopwood (1978) defines the difference between bacteriocins and true antibiotics as genetic - Bacteriocins are the products of single genes, whereas true antibiotics are not encoded by single genes but are the products of multi-enzyme pathways.

Four groups of bacteriocins have so far been identified. Colicins and microcins are produced by members of the *Enterobacteriaceae*, in particular *Escherichia coli* (*E. coli*) (Reviewed by Pugsley, 1984a and b; Kolter and Moreno, 1992). Gram-positive bacteria produce Bacteriocin-Like Inhibitory Substances (BLIS) and lantibiotics which have been the subject of extensive reviews (Tagg *et al.*, 1976; Sahl, 1992; Tagg, 1992; Hansen, 1993; Sahl, 1994; Jack *et al.*, 1995). Colicin V was the first bacteriocin to be

identified, when Gratia described a biologically active factor produced by *E. coli* V which inhibited the growth of *E. coli* ϕ (Gratia, 1925; Fredericq *et al.*, 1949). The early perception of bacteriocin production was typified by the high molecular weight, narrow spectrum of activity, receptor-mediated binding to sensitive cells and production through lethal biosynthesis, which is characteristic of colicin expression. More recently microcins, BLIS (non-lanthionine containing bacteriocins) and lantibiotics have been identified and are similar to colicins in that they are proteinaceous. They have however been classified separately due to a wide activity spectrum, their small size, heat stability, high degree of modifications and non-inducible synthesis (Tagg *et al.*, 1976; Jack *et al.*, 1995; Kolter and Moreno, 1992).

The production and activity of microcins, BLIS and lantibiotics is a multi-step process in both gram-negative and gram-positive bacteria (Figure 1). The producing organism must be able to synthesise the bacteriocin, which involves transcription of the structural gene, translation to form the prepeptide, post-translational modification and processing by cleavage of the N-terminal leader sequence to form the active molecule. Once produced, the cell must be able to protect itself from the bacteriocidal action of the bacteriocin and export the active peptide out of the cell. The bacteriocin must then be able to exert its biological activity on target cells by first interacting with the cell surface and then gaining access to the target site (Kolter and Moreno, 1992).

1.1 BACTERIOCINS PRODUCED BY *ESCHERICHIA COLI.*

1.1.1 COLICINS.

All colicins have three common functional domains and molecular weights ranging from 29kDa to 75kDa (Braun *et al.*, 1994). The catalytic domain is represented by the C-terminal 25% of the molecule which is also involved in interactions with the specific immunity protein, whereas the hydrophobic N-terminal 25% is involved in the translocation of the colicin across the cell membrane. The receptor binding domain is located in the central region of the colicin molecule (Konisky, 1982). Colicin activity can be seen as a single hit phenomenon where one colicin molecule is enough to kill a single sensitive cell (Hardy, 1975) however, not all colicin-receptor interactions lead to cell death (Pugsley, 1984a).

Mode of action.

Colicins are classified by their mode of action and have been divided into various subgroups (Konisky, 1982; Pugsley, 1984a; Luria and Suit, 1986; Braun *et al.*, 1994).

Pore forming colicins (Colicins A, B, E1, Ia, Ib, K, N, S8, 5, 10).

The majority of colicins kill sensitive cells by forming pores in the cytoplasmic membrane, destroying the cell's membrane potential and causing efflux of intracellular K^+ and inorganic phosphates (Lakey *et al.*, 1994). Alignment of the C-terminal catalytic domain has revealed some similarity between these colicins and in particular a long hydrophobic sequence which forms a membrane-spanning hydrophobic hairpin (Pattus, 1990). The membrane inserted state of colicin A has been studied in great depth and was found to be in a "penknife" configuration with the hydrophobic helices 8 and 9 running parallel to the membrane (Lakey *et al.*, 1992; Lakey *et al.*, 1994). For colicin E1 however, it has been proposed that helices 8 and 9 are perpendicular to the membrane (Palmer and Merril, 1994).

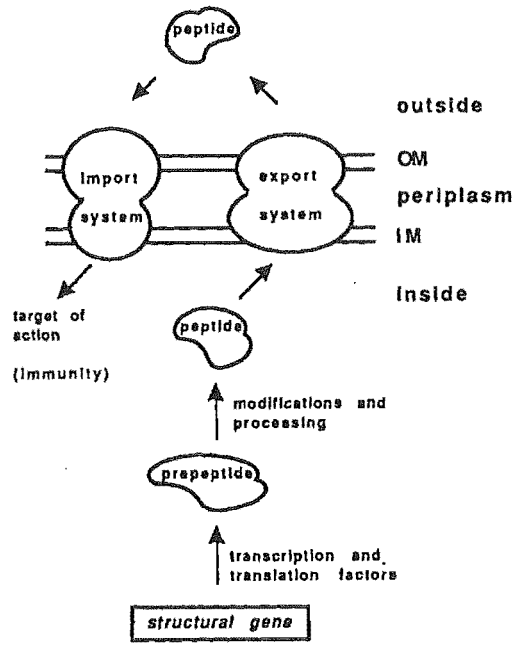


Figure 1. Diagram of the steps involved in peptide antibiotic production and action in gram-negative bacteria. Abbreviations are: OM, outer membrane; IM, inner membrane (From Kolter and Moreno 1992).

Endonucleases.

Colicins with endodeoxyribonucelease activity (colicins E2, E7, E8, E9) kill sensitive cells through the nonspecific degradation of chromosomal DNA. Extensive sequence similarity has been found among these colicins (Toba *et al.*, 1988).

Colicins E3, E4, E6 and cloacin DF13 all possess endoribonuclease activity. Cell death is caused by the inhibition of protein synthesis, brought about by an endonucleolytic break that cleaves a 49 nucleotide fragment from the 3'-OH end of the 16sRNA molecule within the 30s ribosomal subunit. It also appears that the 50s ribosomal subunit plays a role in the activity of these colicins (Luria and Suit, 1986). Extensive protein homology is found in the catalytic domains of these colicins (de Graaf and Oudega, 1986) but not between these and the endodeoxyribonuclease colicins (Pugsley, 1984a; Cole *et al.*, 1985).

Inhibition of peptidoglycan biosynthesis.

Colicin M is the only colicin known to inhibit peptidoglycan biosynthesis through the inhibition of bactoprenyl-P carrier lipid regeneration (Harkness and Ölschläger, 1991). The active site in sensitive cells is only accessible to colicin M from the periplasmic side of the inner membrane (Ölschläger, 1991).

Other mechanisms.

Both colicins D and E5 inhibit protein synthesis but possibly by a different mechanism than RNase activity. The mechanism of activity for colicins G and H is unknown.

Genetics.

The genes for colicin activity, immunity and release are encoded on plasmids (Hardy, 1975; Luria and Suit, 1986), with the exception of colicin L, a chromosomally-encoded colicin produced by *Serratia marcescens* JF246 (Pugsley, 1984a). Two groups of Col plasmids have been distinguished and individual colicins are encoded on one type, never both. Group I Col plasmids are multicopy plasmids with a molecular weight of $3x\ 10^6$-$6x10^6$, whereas group II Col plasmids are large, often conjugative, low copy number plasmids with a molecular weight of between $7x10^7$-$12x10^7$ (Luria and Suit, 1986).

Three genes have been identified among the group I Col plasmids which encode the structural, immunity and lysis proteins. The genetic arrangement of these genes is similar for all colicins (Figure 2) and transcription is under the control of an inducible promoter lying upstream of the structural gene. The immunity gene of pore forming colicins is transcribed from its own promoter in the opposite direction to that of the structural and lysis genes. As a result, transcription of the lysis gene is inhibited, causing a build up of cytoplasmic colicin prior to release (Pugsley, 1984a; Luria and Suit, 1986; Braun *et al.*, 1994).

The release of colicins encoded by group I Col plasmids is a lethal process for the producing cells, resulting in partial lysis of the culture and semi-selective release of the colicin from the producing cells. Killing of the producing cells and activation of phospholipase A are induced by the lysis protein, a small lipoprotein encoded by the lysis gene (de Graaf and Oudega, 1986; Lazdunski *et al.*, 1988; Lakey *et al.*, 1994). Lysis proteins are translated with a 17-19 amino acid signal sequence and share extensive sequence similarity to each other (Cavard and Oudega, 1992). The activation of phospholipase A by the lysis protein may play an important role in the release of the colicin through destabilisation of the cell membrane (Pugsley, 1984a; de Graaf and Oudega, 1986). The mechanism of colicin release encoded by group II Col plasmids is unclear since they do not encode lysis proteins and are inefficiently released (Pugsley, 1984a).

Colicin specific immunity genes are also encoded on Col plasmids and form the basis on which novel colicins are identified from new *E. coli* isolates (Pugsley, 1985). By definition, immunity is the property by which colicinogenic cells are not killed by the particular colicin that they produce (Jakes and Lazdunski, 1992).

The immunity protein for endonuclease-acting colicins is made in a stoichiometric 1:1 proportion to that of the colicin, protecting the cell by binding to the catalytic domain of the colicin (Pugsley, 1984a). Commonality in amino acid sequence has been found among the immunity proteins of enzymatic colicins regardless of their DNase or RNase activity (de Graaf and Oudega, 1986). The exact nature of the interaction between the immunity protein and catalytic domain remains unclear, however the colicins remain bound to their respective immunity proteins upon release

and are dissociated after binding to the receptors on a target cell (Jakes and Lazdunski, 1992).

Immunity to pore forming colicins functions through the direct interaction of the immunity protein with the pore forming domain of the respective colicin at the cell membrane, resulting in the prevention of pore formation (Géli and Lazdunski, 1992). The immunity protein is only required to protect the producing cell from external colicin since the transmembrane potential is the opposite of that required for colicin activity (Jakes and Lazdunski, 1991). The addition of excess extracellular colicin results in a phenomenon called immunity breakdown (Konisky, 1982, Pugsley, 1984b), whereby insufficient immunity protein exists to bind all of the colicin molecules within the cell membrane (Lakey *et al.*, 1994). Classification of immunity proteins is based on the number of transmembrane domains, resulting in the proper protein topology. The first group has four transmembrane domains typified by the ColA immunity protein and the second, like the ColE1 immunity protein, has three transmembrane domains (Géli and Lazdunski, 1992).

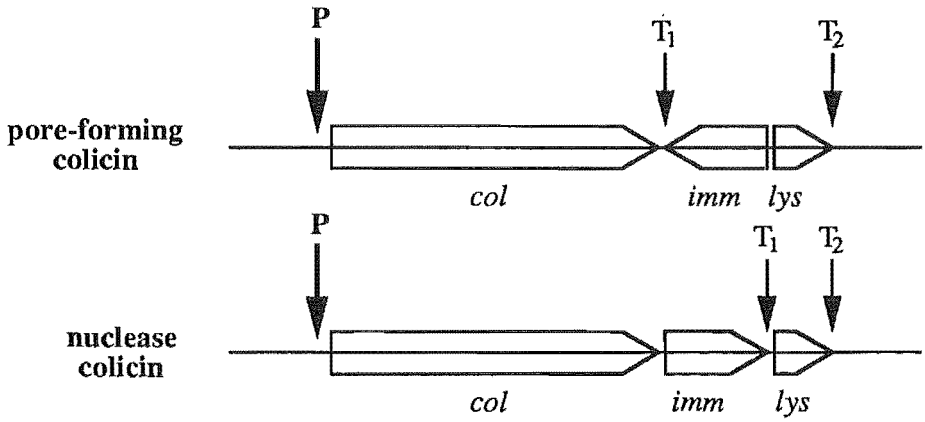


Figure 2. Genetic organisation of colicin operons. *col*, colicin structural gene; *imm*, colicin immunity gene; *lys*, colicin lysis gene; P, promoter; T, transcription terminator (From Cavard and Oudega, 1992).

Colicin production is a highly inducible process controlled by the SOS response, a system that regulates gene expression after DNA damage through the relief of LexA repression by the RecA protease mediated proteolytic cleavage of LexA (Braun *et al.*, 1994). Once repression is removed amplification of colicin synthesis by several orders of magnitude occurs (Luria and Suit, 1986), with only a small proportion of cells in a population expressing the colicin genes thereby maximising selection against non plasmid bearing cells (Lakey *et al.*, 1994). Binding sites for the LexA repressor are found within the structural gene promoter of all colicins regulating the expression of the structural, immunity and lysis genes (Luria and Suit, 1986; de Graaf and Oudega, 1986). Immunity genes which are transcribed in the opposite orientation to that of the structural gene are not SOS regulated and have their own weak promoter (Braun *et al.*, 1994). Catabolite repression may also play a role in the regulation of colicin expression (Pugsley, 1984a).

Receptor binding and translocation.

The activity spectrum of a particular colicin is defined by the distribution of specific receptors on sensitive cells. This interaction is an energy independent, trypsin sensitive process. Different colicins may share the same receptor, recognising the same or different receptor domains (Pugsley, 1984a). Colicin receptors also have normal physiological roles in the life of the cell. Many serve as nutrient uptake receptors such as ButB, the receptor for the E colicins which also is involved in vitamin B_{12} uptake and FepA (colicin B) or Cir (colicins Ia and Ib) which are involved in iron-enterochelin binding and iron accumulation, respectively (Pugsley, 1984a). The dependence of colicins on receptors which are required for normal cellular activities has been proposed as a method by which continued selective pressure has maintained the presence of the receptor required for colicin uptake (Konisky, 1982).

Once bound to the receptor, an energy dependent translocation of the colicin to a trypsin inaccessible state can occur by two pathways, classifying colicins into two groups dependent on which pathway is used. Mutations in genes in either pathway result in colicin tolerance in the mutant strain (Pugsley, 1984a). TonB, the product of the *tonB* gene, is an energy transducer, coupling energy to the active transport of iron siderophores and vitamin B_{12} (Postle, 1990; Kadner, 1990) and defines the translocation

of the group B colicins (B, Ia, Ib, M, D). Proteins and their receptors which utilise TonB all contain a sequence at their N-terminus designated the "TonB box" which is the site of interaction with TonB (Braun *et al.*, 1994). Group A colicins (E1, E2, E3, A, K, L, DF13, N, S4) are defined by utilising the products of the *tolQRAB* genes for translocation by an energy independent process. Colicin E1 is an exception since it strictly requires TolC and not TolB (Braun *et al.*, 1994). The *tolA* gene product is analogous to TonB, however the specific role of the *tol* genes is unknown except they play a role in the import of certain biologically active macromolecules (Webster, 1991). Colicins which use this pathway contain a glycine rich N-terminal region not found in their receptors which has been designated the "TolA box" (Pilsl and Braun, 1995).

Two recently studied colicins have been found to utilise components of both the Ton and Tol systems. Colicins 5 and 10 both bind to the Tsx protein, the receptor for colicin K which utilises the Tol system, however colicins 5 and 10 use the TonB system (Bradley and Howard, 1992; Pilsl and Braun, 1995). Colicin 10 is the first colicin to require both TonB and TolC for translocation (Pilsl and Braun, 1995).

1.1.2 MICROCINS.

Asensio *et al.* (1976) were the first to identify a class of low molecular weight antibiotics, produced by enteric bacteria isolated from the faeces of newborn infants, which could inhibit the growth of related microorganisms. These antibiotics were able to diffuse through cellophane, inhibit the growth of an indicator bacterium and were found to be soluble in methanol-water (5:1) and thermostable. The generic name "microcin" was proposed to differentiate these antibiotics from colicins.

Microcins are low molecular weight (<10 kDa) ribosomally synthesised peptide antibiotics, produced by diverse members of the *Enterobacteriaceae* and are active against *E. coli* and other enteric bacteria. Like colicins, the microcins are proteinaceous, except for MccA15 which is a methionine analogue (Aguilar *et al.*, 1982) and are produced in association with a specific immunity protein. Microcin production however, is not SOS inducible but occurs at the start of stationary phase growth. Synthesis is non-lethal for the producing strain and requires a dedicated export system for secretion (Reviewed by Baquero and Moreno, 1984; Kolter and Moreno, 1992). Uptake and translocation into sensitive cells, as with the colicins, is receptor mediated

(Pugsley, 1984a). The microcin structural, immunity and export genes are encoded on plasmids (Baquero *et al.*, 1978), with the exception of MccH47 which is chromosomally encoded (Laviña, 1990). Unlinked chromosomal genes are also known to be involved with the post-translation modification and export of microcins (Kolter and Moreno, 1992).

Microcin classification was standardised by Baquero and Moreno (1984). Based on cross immunity tests between producing strains, five phenotypic groups (I-V) were established. Microcin types A-E were then proposed for the activities related to the plasmids found in the phenotypic groups I-V, with type A microcins inhibiting metabolic enzymes, type B microcins inhibiting DNA replication, type C microcins affecting protein synthesis and type D microcins impairing the cells energy system (Table 1). Subsequently a type H microcin has been identified but the mechanism of action is unknown (Laviña, 1990).

Survey of microcins: Genetics, activity and regulation.

The emphasis of this survey will be on ColV, MccB17 and MccC7 because of their relevance to the current work.

Colicin V.

Historically this toxin has been classified as a colicin. However, genetic and functional analysis has shown that ColV is a small peptide, the synthesis of which is not SOS-inducible and the release of which is by a dedicated export system. These characteristics have meant that ColV is now considered a microcin (Fath *et al.*, 1992). Many similarities have also been found between ColV and the non-lanthionine containing bacteriocins produced by gram-positive bacteria (Håvarstein *et al.*, 1994).

The biological activity of ColV is directed against the cell membrane of sensitive cells, disrupting the membrane potential (Yang and Konisky, 1984), however not through the formation of channels (Fath *et al.*, 1991). ColV synthesis and uptake is iron regulated. Transcriptional regulation of the ColV genes is controlled by the Fur repressor encoded by the *fur* gene; increased ColV production occurs when iron availability is low and derepression in iron rich media occurs in *fur* mutants. Uptake of ColV into sensitive cells requires the Cir protein as the outer membrane receptor and

TonB which is responsible for translocation of the toxin. Both genes are also involved in iron accumulation (Chehade and Braun, 1988).

Table 1. Classification and activity of known microcins.

Mode of action	Microcin*	Reference.
Dissipation of proton motive force	ColV	Yang and Konisky, 1984
	MccE492	de Lorenzo and Pugsley, 1985
	MccD140	Duro *et al.*, 1979
	MccD15	Aguilar *et al.*, 1983
Inhibition of cell division	Mcc25	Salomón and Farías, 1992
Disruption of DNA biosynthesis	MccD93	Martínez and Pérez-Díaz, 1986
Inhibition of DNA replication	MccB17	Herrero and Moreno, 1986; Vizán *et al.*, 1991
Blockage of protein biosynthesis	MccA15, MccA93 and MccA136	Aguilar *et al.*, 1982; Pérez-Díaz and Clowes, 1980
	MccC7	Garcia-Bustos *et al.*, 1985
Prevention of precursor inclusion (DNA, RNA, protein)	MccC51	Kurepina *et al.*, 1993
Unknown	MccH47	Laviña *et al.*, 1990
Unknown	MccB2 and MccB27	Basyuk *et al.*, 1994
Unknown	MccR51	Kurepina *et al.*, 1992
Unknown	MccJ	Martin, 1994; Kippenberger, 1996
Nonspecific nuclease	Mcc24	This work

*Group classification is specified by the letter after Mcc. All strains producing group A microcins also produce a group D microcin, encoded by a different plasmid (Baquero and Moreno, 1984).

The genes encoding ColV production, immunity and export are encoded on a number of large, conjugative plasmids which have been associated with the pathogenicity of *E. coli* (Reviewed by Waters and Crosa, 1991). Frick *et al.* (1981) were the first to clone the structural and immunity genes in a 900bp fragment from pColV-B188 however, this fragment failed to produce extracellular ColV. The complete genetic region was cloned from pColV-K30 into pBR322 by Gilson *et al.* (1987). Through mutagenesis and complementation, the cloned 9.4kb *Hin*dIII-*Sal*I fragment was found to encode four genes within a 4.4kb region: *cvaC*, the structural gene; *cvi,* encoding specific immunity; *cvaA/cvaB* required for extracellular secretion of the toxin. Sequence analysis revealed that the four genes form two converging operons comprised of *cvaC/cvi* and *cvaA/cvaB* (Gilson *et al.*, 1990; Figure 3).

Cvi.

The Cvi protein is a 78 amino acid inner membrane protein which is sufficient for ColV specific immunity (Fath *et al.*, 1992). Cytoplasmic ColV is not bacteriocidal to the producing cell, therefore the role of Cvi is to protect the producing cells from ColV molecules interacting with the inner membrane from the periplasmic side (Zhang *et al.*, 1995).

CvaC.

The ColV protein, CvaC, was visualised by SDS-PAGE as a 6kDa protein (Gilson *et al.*, 1987). Sequence analysis of the *cvaC* gene has predicted a 103 residue protein with a molecular mass of 10.304kDa (Gilson *et al.*, 1990), indicating that ColV might be subject to post-translational modification. Processing of CvaC involves the cleavage of the 15 N-terminal amino acids, termed the leader sequence or double glycine type leader peptide, resulting in an 88 amino acid mature peptide with bacteriocidal activity (Fath *et al.*, 1994; Håvarstein *et al.*, 1994). The leader peptide is required for signal sequence-independent export of ColV, involving the transport proteins CvaA and CvaB (Zhang *et al.*, 1995). Fath *et al.* (1994) identified the C-terminal region as possessing the bacteriocidal activity which was retained after SDS-PAGE resulting in an active band of 5.8kDa. It has been suggested that further non-covalent modifications of the ColV peptide occur after cleavage, resulting in a possible metalloprotein with iron (Fath *et al.*,

1994), or a possible disulphide bridge between the C-terminal cysteine residues at positions 91 and 102 (Håvarstein *et al.*, 1994). The increased stability of the protein after either modification results in an unusual mobility under SDS-PAGE and the low estimation of molecular mass by this method.

The leader peptide of pre-ColV shares many similar features with non-lanthionine containing bacteriocins and some lantibiotics produced gram-positive bacteria, making ColV the first gram-negative bacteriocin to fit into this group. Protein sequence alignment has revealed many features common to this class of bacteriocin: (a) a cleaved leader sequence of 15-12 amino acids; (b) N-terminal sequence of Met-(Lys/Arg); (c) a predicted α-helical structure over most of the leader sequence; (d) a negatively charged region in the middle of the leader sequence; (e) a predicted turn starting 1-3 residues from the cleavage site which is proposed to expose the cleavage site to the protease; (f) cleavage following a Gly-Gly pair; (g) processing results in a small bacteriocin of between 43-88 amino acids; (h) export of the bacteriocin via an ABC transport complex (Fath *et al.*, 1994; Håvarstein *et al.*, 1994).

Export of ColicinV: CvaA, CvaB and TolC.

The extracellular secretion of ColV is a signal sequence independent process requiring the products of three genes, *cvaA*, *cvaB* and the chromosomally encoded gene *tolC*, which together form a dedicated export complex (Gilson *et al.*, 1990; Figure 3). Zhang *et al.* (1995) have shown that a small amount of active ColV is present in the periplasm during export. Pre-ColV was found to be very unstable in the absence of CvaA and CvaB, with CvaB being an absolute requirement for processing.

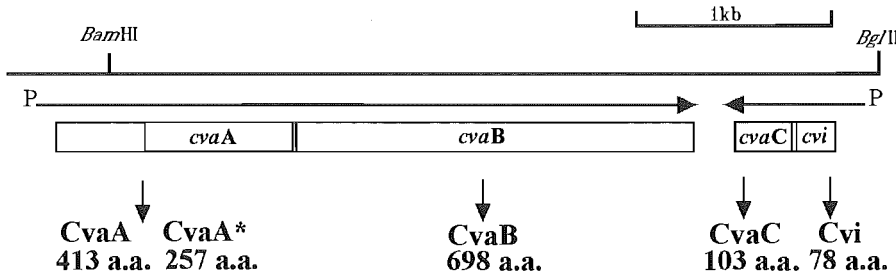
CvaA is a member of the membrane fusion protein (MFP) family which are accessory proteins required in gram-negative bacteria when the substrate is exported across both membranes (Dinh *et al.*, 1994). It has been speculated that MFP proteins may form bridges between the two membranes (Skvirsky *et al.*, 1995). DNA sequencing and minicell protein analysis has identified two forms of CvaA, a full length 413 amino acid protein (47kDa) and a truncated 256 amino acid (27kDa) protein designated CvaA*, the result of a translational restart at the in-frame methionine codons at positions 156 and 160 (Gilson *et al.*, 1990). CvaA is an inner membrane protein,

anchored to the membrane by a single N-terminal domain while the majority of the protein is located in the periplasm (Skvirsky *et al.*, 1995).

DNA sequence analysis of *cvaB*, revealed that CvaB is a member of the MDR-like (Multiple Drug Resistance) or ABC transporter (ATP Binding Cassette) family of proteins (Gilson *et al.*, 1990), characterised by a highly conserved ATP-binding domain at the C-terminus end of the protein. These proteins hydrolyse ATP to provide the energy for active transport of the substrate (Fath and Kolter, 1993). CvaB is a 698 amino acid (78kDa), integral inner membrane protein containing six transmembrane domains between residues 179-438 (Gilson *et al.*, 1990; Fath *et al.*, 1992). The cytoplasmic C-terminal domain shares the highest sequence similarity with other ABC transporters and contains the ATP binding fold consisting of an A and B site (Fath *et al.*, 1992). Functional complementation between the exporters CvaAB, HylBD (α-hemolysin) and PrtDEF (*Erwinia* protease) has been demonstrated. ColV was able to be exported by HylBD and PrtDEF in the absence of CvaAB but at a lower efficiency. Complementation was not reciprocal and hemolysin was unable to be exported by CvaAB or PrtDEF. Differences in signal specificity for CvaC was also found between the CvaAB and HylBD export systems (Fath *et al.*, 1991).

TolC is a minor outer membrane protein, first identified as being required for the excretion of α-hemolysin (Wandersman and Delepelaire, 1990). Gilson *et al.* (1990) found that TolC was also required for ColV secretion. Export was reduced in *tolC* mutants and could be restored by the transformation of plasmids encoding the *tolC* gene.

A.



B.

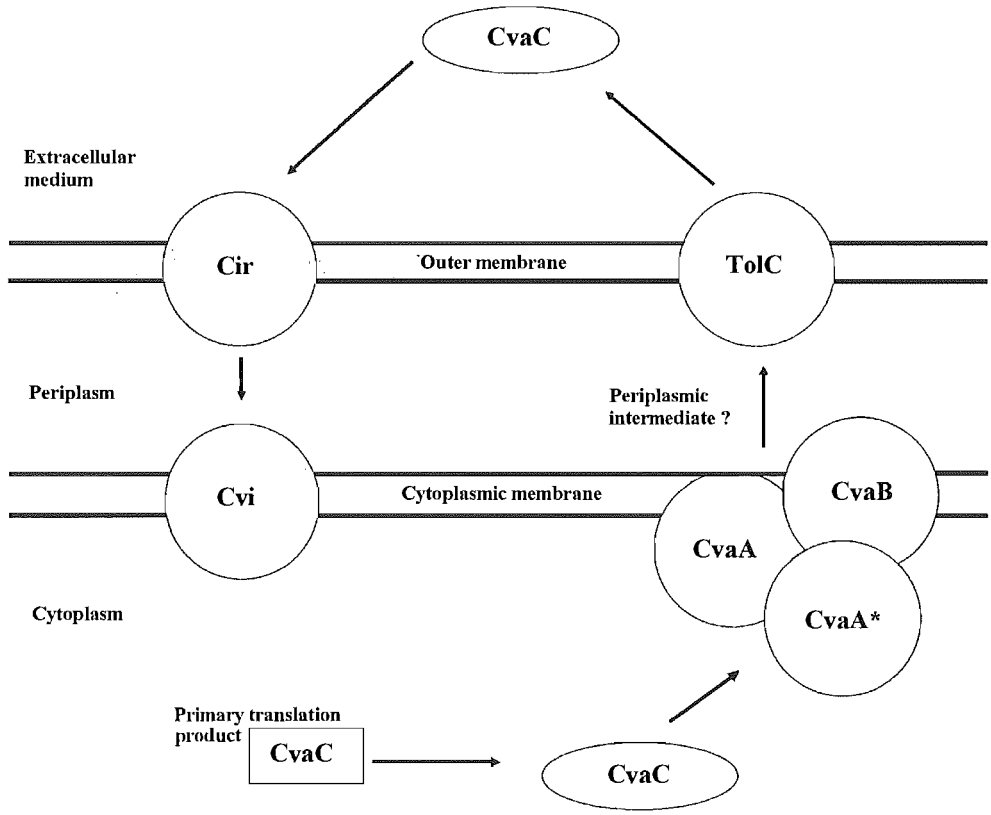


Figure 3. Molecular biology of colicin V. A. Genetic organisation of the colicin V operons (From Gilson *et al.*, 1990). Amino acids (a.a.). **B.** The current model for colicin V production, export, immunity and activity (From Fath *et al.*, 1992).

Microcin B17.

Microcin B17 was the first peptide antibiotic shown to inhibit a type II DNA topoisomerase. The primary effect of this microcin is to inhibit DNA elongation by blocking DNA gyrase at the replication fork, trapping an enzyme-DNA cleavable complex. This results in a double stranded cleavage of the DNA mediated by DNA gyrase, inducing the SOS repair system and causing massive DNA degradation (Herrero and Moreno, 1986; Vizán *et al.*, 1991; Hernández-Chico *et al.*, 1992).

The genes encoding MccB17 were first cloned from the 70kb conjugative plasmid pMccB17 (pRYC17) to a 5.1kb fragment (San Millán *et al.*,1985a). Complementation of Tn*5* mutants within this region revealed four loci, termed *mcbABCD,* which were essential for MccB17 production (San Millán *et al.*, 1985b) and DNA sequencing and transcriptional analysis identified three promoters (Genilloud *et al.*, 1989). Analysis of the region downstream of *mcbD* located three more genes, *mcbEFG* (Figure 4). It was found that immunity to endogenous MccB17 occurred by two mechanisms. Firstly, the genes *mcbEF* encoded an export complex which rapidly exported active MccB17 out of the producing cell. Sequence analysis indicated that McbF was a member of the ABC family of transport proteins. The specific immunity to endogenous MccB17 was provided by the product of the *mcbG* gene (Garrido *et al.*, 1988). Herrero *et al.* (1986) found that immunity due to McbG does not neutralise the toxic activity, but rather prevents the subsequent events leading to cell death after the inhibition of DNA synthesis, but before DNA degradation begins. Recently, Baquero *et al.* (1995) have described a chromosomal gene, *sbmC*, which when cloned into a high copy vector results in high levels of resistance to exogenous MccB17. Induction of *sbmC* is controlled by SOS regulation and stationary phase growth. It is suggested that SbmC recognises and sequesters MccB17 in a reversible way since it confers resistance and blocks MccB17 export from producing cells.

The structural gene for MccB17 was identified as *mcbA*, encoding a 69 amino acid primary translation product. This product is subject to post-translational modification and processing prior to export, resulting in an active microcin of 43 amino acids, of which 23 were glycine residues (Davagnino *et al.*, 1986). Two mechanisms of post-translational modification were determined for the maturation of MccB17 (Figure

5a). The 69 amino acid pre-MccB17 peptide is first modified and folded, a process controlled by McbBCD (Yorgey *et al.*, 1993). Chemical modification of four cysteine and four serine residues resulting in four thiazole and four oxazole rings which in turn fold to produce two aromatic heterocycles (Bayer *et al.*, 1993; Yorgey *et al.*, 1994). This proMccB17 form is biologically active but is unable to be exported out of the cell (Rodriguez-Sáinz *et al.*, 1990). The final processing step involves the removal of the first 23 N-terminal amino acids, termed the leader peptide, to produce the active MccB17. Rodriguez-Sáinz *et al.* (1990) identified a chromosomal gene at 96 minutes on the *E. coli* chromosome map, called *pmbA*, which encoded the cytoplasmic peptidase responsible for the cleavage. Once exported, MccB17 gains entry into sensitive cells through binding to OmpF, an outer membrane protein. Translocation into the cell is mediated by SbmA, an inner membrane protein encoded by *sbmA* (Laviña *et al.*, 1986; Liu, 1994).

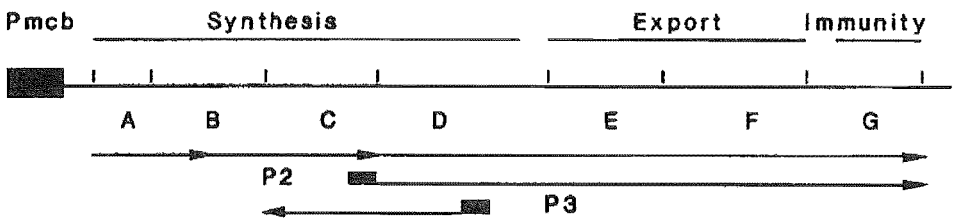
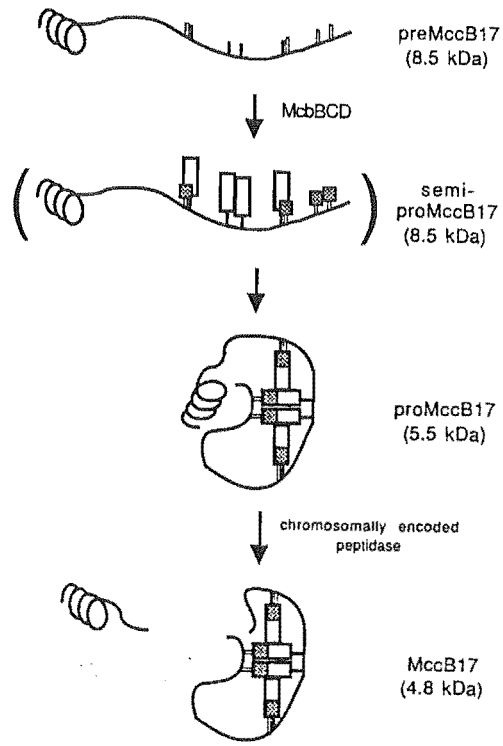


Figure 4. Organisation of the microcin B17 genetic system (From Moreno *et al.*, 1992).

A.



B.

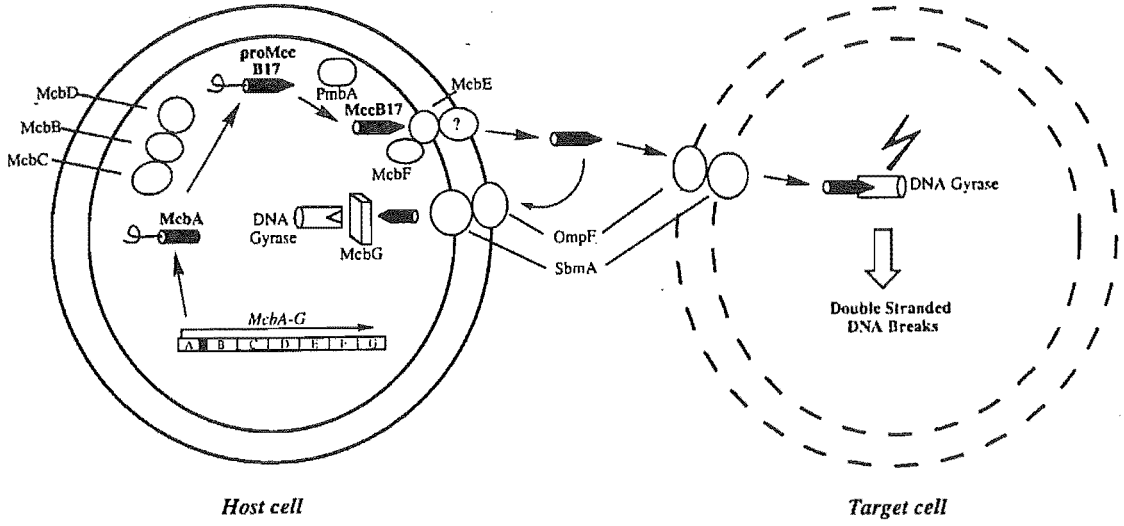


Figure 5. Biosynthesis of microcin B17. A. Steps in MccB17 maturation. Schematic representations: solid vertical bars on preMccB17 = cysteine residues; open vertical bars on preMccB17 = serine residues; open rectangles on solid bars = modified serine residues. Molecular mass values in parenthesis were determined by relative mobility in SDS-PAGE (From Yorgey *et al.*, 1993). **B.** Biosynthesis and mechanism of action of MccB17 (From Liu, 1994).

Expression of MccB17 occurs as the cell enters stationary phase growth and is regulated by several chromosomally encoded genes (Figure 6; Hernández-Chico *et al.*, 1986; Kolter and Moreno, 1992). Growth phase regulation of *mcbABCDEFG* transcription is controlled from the promoter P_{mcb} located upstream of *mcbA*. Two non-regulated promoters have also been found within *mcbC*, one promoting low level expression of *mcbD* and the other directing transcription in the opposite direction to that of P_{mcb} (Hernández-Chico *et al.*, 1982; Genilloud *et al.*, 1989). Growth phase dependent activation of P_{mcb} was found to be controlled by the transcription activator OmpR (Hernández-Chico *et al.*, 1982; Hernández-Chico *et al.*, 1986). The IHF protein, encoded by the chromosomal genes *himA* and *himD* also activates transcription from P_{mcb}, possibly through DNA bending (Moreno *et al.*, 1992). Negative regulation of MccB17 expression is mediated by the products of the chromosomally encoded genes *mprA* (del Castillo *et al.*, 1990) and *bglY* (*hns*) encoding the histone like protein H-NS (Moreno *et al.*, 1992).

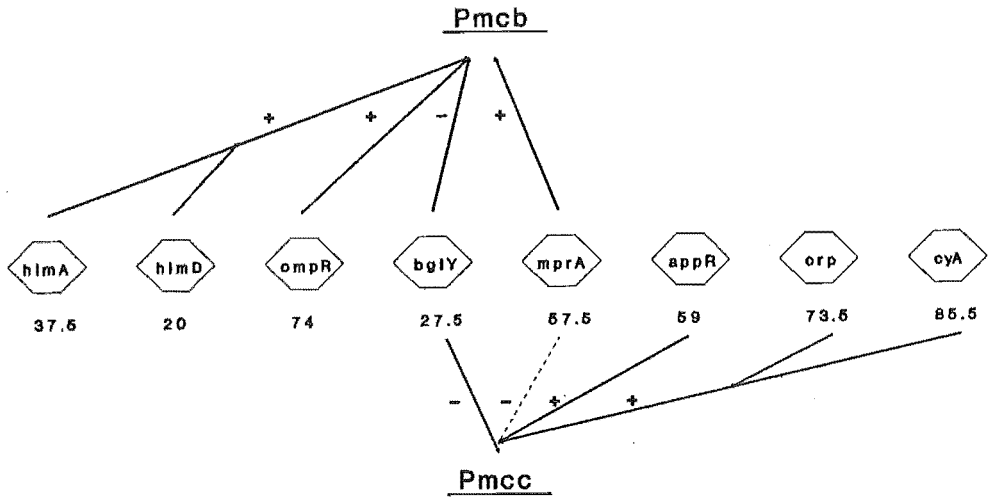


Figure 6. ***Escherichia coli*** **chromosomal genes regulating P_{mcb} and P_{mcc}.** (+) indicates activation of promoter; (-) inhibition of promoter. The dashed line indicates that the P_{mcc} is clearly inhibited only in the presence of many copies of *mprA*. Numbers under the genes indicate their location on the genetic map (From Moreno *et al.*, 1992).

Microcin C7.

Microcin C7 is a linear heptapeptide which inhibits cellular protein synthesis. The genes for MccC7 are encoded on a 43kb plasmid pMccC7 (Garcia-Bustos *et al.*, 1984; Garcia-Bustos *et al.*, 1985). Novoa *et al.* (1986) cloned two adjacent *Hin*dIII fragments from pMccC7 which encoded production and immunity. Complementation analysis identified four DNA regions named α, β, γ and δ, all of which were transcribed in the same direction and all necessary for microcin production. The gene *mccA* was identified as the gene encoding MccC7 and contained a 21bp coding sequence for the seven amino acids of the microcin (González-Pastor *et al.*, 1994). Chemical analysis of MccC7 revealed that the peptide was subject to post-translational modification at the N and C termini. The N-terminus possessed a N-formyl group whereas the C-terminus contained an aspartic acid residue, the product of deamidation of the DNA-encoded arginine, connected via an amide to a modified nucleotide (5' adenylic acid) (Guijarro *et al.*, 1995). Experiments showed that the peptide was the biologically active portion of the molecule and the C-terminal substituent has a role in transport of MccC7 into sensitive cells.

Gonzáles-Pastor *et al.* (1995) have recently sequenced the entire MccC7 coding region and identified six clustered genes responsible for MccC7 production and immunity, *mccABCDE* and *mccF* (Figure 7A). Transcription of the *mccABCDE* operon was initiated from the promoter P_{mcc} (*mccp*) whereas *mccF* was transcribed from its own promoter in the opposite direction. Immunity to MccC7 was mediated by MccC, responsible for rapid export of MccC7, MccE which appeared to be a cytosolic protein and possibly acetylated the antibiotic target on the ribosome, and MccF which appeared to sequester MccC7 in the periplasm. Post-translational modification of proMccC7 was proposed to be mediated by MccB and MccD, with MccB responsible for the C-terminal adenylation (Figure 7B).

Expression of MccC7 occurs as the cells enter stationary phase (Figure 5). Diaz-Guerra *et al.* (1989) found that growth phase dependent transcription from P_{mcc} was controlled by the *appR* gene, also called *katF* or *rpoS*. AppR was not required for expression but ensured full expression from the promoter (Moreno *et al.*, 1992).

Expression of MccC7 was also found to be subject to catabolic activation through the activity of the CRP protein (cAMP receptor protein) (Moreno *et al.*, 1992).

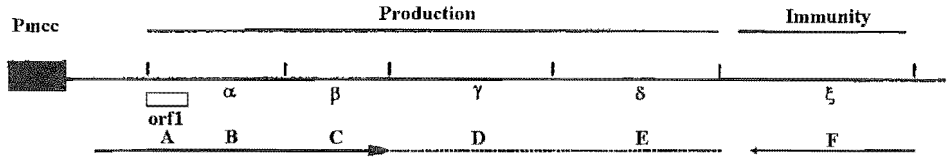
Microcin E492.

Microcin E492 is a 6kDa hydrophobic protein, produced by a faecal isolate of *Klebsiella pneumoniae* and is active against *E. coli, Klebsiella, Salmonella, Citrobacter, Enterobacter* and *Erwinia* species but not *Shigella, Proteus, Serratia* or *Pseudomonas* species (de Lorenzo, 1984). Sensitive cells are killed by the depolarisation of the cytoplasmic membrane through the formation of cation selective channels (de Lorenzo and Pugsley, 1985; Lagos *et al.*, 1993). Uptake of MccE492 requires the *tonB* and *exbB* genes as well as the product of the *semA* gene, which function as the outer membrane receptor (Pugsley *et al.*, 1986). Little is known about the genetics of MccE492 production. The genes may be located on the chromosome or on a large non-mobilisable plasmid. The two visible plasmids within the strain were found not to encode MccE492 production (de Lorenzo and Pugsley, 1985).

Microcin H47.

Microcin H47 is the first microcin whose genetic system has been found to reside on the chromosome of producing cells. Produced by a naturally occuring isolate, *E. coli* H47, this microcin is active against *E. coli, Salmonella, Enterobacter, Shigella, Klebsiella* and *Proteus* species but not against gram-positive species. The genes encoding MccH47 were found to reside on a 10kb chromosomal fragment (Laviña *et al.*, 1990; Laviña and Gaggero, 1992). Complementation analysis has revealed that six genes are required for production, *mchA-F*, with *mchABCD* being required for production and *mchEF* for export. The immunity gene, *mchI* lies in a 750bp fragment upstream of *mchB*. An unusual feature is a 3kb silent region between *mchA* and *mchB*. Mutations and deletions within this region do not seem to alter microcin production, however a gene *mchS1* has been identified within this region which encodes a 43.5kDa protein. Similarities between *mchE* and *cvaA* have been found since both encode two translation products (Gaggero *et al.*, 1993).

A.



B.

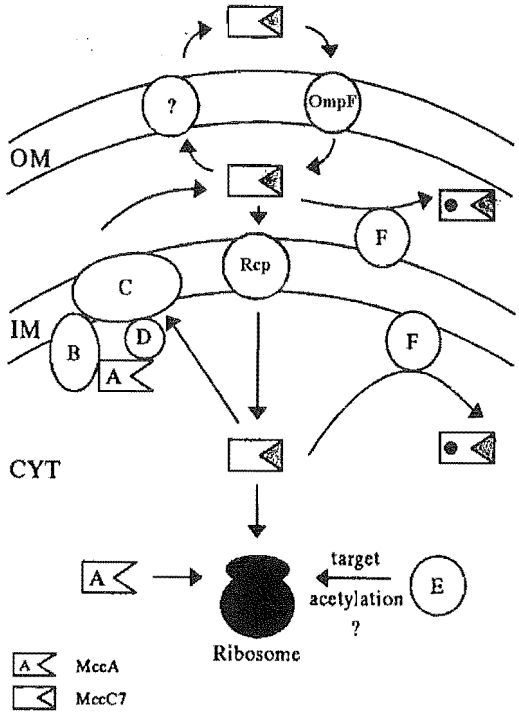


Figure 7. Genetic organisation and production of microcin C7. A. Structure of MccC7 genetic system. ORF1 indicates the open-reading frame that encodes the primary structure of MccC7 (From Moreno *et al.*, 1992 and Gonzáles-Pastor *et al.*, 1995). **B.** Model for MccC7 production and immunity. The gene products MccA to F are indicated by A to F respectively. Rcp designates a presumed MccC7-specific receptor on the inner membrane (IM); OmpF is an *E. coli* porin on the outer membrane (OM) shown to be involved in MccC7 uptake (From Gonzáles-Pastor *et al.*, 1995).

Microcins A15, A93 and A136.

Microcins A15, A93 and A136 are plasmid encoded and produced by *E. coli* strains LP15, LP93 and LP136 respectively. It is thought that these microcins are the same since *E. coli* LP15, LP93 and LP136 have cross-immunity to each other, and *E. coli* LP15 and LP93 share the same 3.9-Md plasmid and *E. coli* LP136 possesses a plasmid with 2.4-Md of homology to the 3.9-Md plasmid (Pérez-Díaz and Clowes, 1980). These three microcins all inhibit protein synthesis in sensitive cells. The mechanism of MccA15 action has been studied and was found to inhibit the enzyme homoserine-O-transsuccinylase, an enzyme involved with L-methionine biosynthesis (Aguilar *et al.*, 1982).

Microcin C51.

Microcin C51 is a novel microcin produced by a faecal isolate of *E. coli* which inhibits a wide range of gram-negative species and some gram-positive species by preventing incorporation of RNA, DNA and protein precursors (Kurepina *et al.*, 1993). The genes coding for MccC51 production and immunity reside on a 38kb conjugative plasmid and at least three genes are required for production of MccC51 and two for immunity (Kurepina *et al.*, 1993). The structure of MccC51 is very similar to that of MccC7, both sharing the same heptapeptide and cross immunity. Metlitskaya *et al.* (1995) have suggested that MccC51 contains an asparagine linked with nebularin-5'-monophoshate at the C-terminus, however their interpretation of the data has been disputed, with the suggestion that MccC51 and MccC7 may in fact be the same (Guijarro *et al.*, 1995). The chromosomally encoded *ompR* gene has been found to be required for MccC51 synthesis (Kurepina *et al.*, 1993).

Microcin 25

Microcin 25 is a plasmid encoded microcin which interferes with cell division resulting in the filamentation of sensitive cells. The activity spectrum of Mcc25 is restricted to *E. coli, Salmonella* and *Shigella*. Analysis of the amino acid composition has revealed that Mcc25 is made up of 20 amino acids (2.1kDa) of which five are glycine. The molecule is hydrophobic with a blocked N-terminus (Salomón and Farías, 1992). Induction of

Mcc25 synthesis occurs at the start of stationary phase growth and is regulated by iron however, this regulation appears to be independent of the Fur repressor (Salomón and Farías, 1994). The uptake of Mcc25 shares many features with other colicins and microcins including the receptor, FhuA, a multifunctional outer-membrane receptor for ferrichrome which is also the receptor for ColM, ExbBD and TonB for translocation into the cell and the *sbmA* gene product which is also required for MccB17 uptake (Salomón and Farías, 1993 and 1995).

Type D Microcins.

Three microcins belong to this group; MccD15, MccD93 and MccD140. It has been proposed that MccD15 and MccD93 are the same or very similar and are closely related to MccD140. Differences in their reported modes of action have meant that biochemical evaluation is needed to clarify their relatedness (Martínez and Pérez-Díaz, 1986).

Microcin D93 is a small hydrophilic peptide with a molecular weight of less than 1kDa. Sensitive strains treated with MccD93 suffer a reduction in the rate of DNA biosynthesis, an effect increased in *recA* strains. Sensitivity to MccD93 is found among *E. coli*, *Proteus, Citrobacter* and *Pseudomonas* species (Martínez and Pérez-Díaz, 1986). Production and immunity of MccD93 is encoded on a 5.5kb nonconjugative plasmid, pMccD93. Cloning of the determinants for microcin production has revealed a 2.3kb region which encoded MccD93 production but only has low levels of immunity, suggesting that not all of the immunity determinants were cloned. The small size of this region implies a more simple genetic arrangement than that found in other microcin genetic systems (Martínez and Pérez-Díaz, 1990).

MccD15 is a 500 dalton polar peptide encoded by a 3.7 Md conjugative plasmid (Aguilar *et al.*, 1982). Both MccD15 and MccD140 target the inner membrane of sensitive cells, disrupting the proton motive force (Aguilar *et al.*, 1982; Duro *et al.*, 1979).

Microcin R51

Microcin R51 is a plasmid encoded microcin produced by *Citrobacter* R51 which is active against a wide variety of gram-negative and gram-positive species (Kurepina *et al.*, 1990). A 5kb fragment was found to encode microcin production and immunity with full immunity requiring 4.6kb and partial immunity 1.9kb (Kurepina *et al.*, 1992).

Microcins B2 and B27.

Microcin B2 production and immunity are encoded on a 4.2kb and 1.4kb DNA fragment respectively. A 5kb fragment was found to be necessary for MccB27 production and immunity as well as the product of the *ompR* gene. Susceptibility to both microcins was increased when the sensitive cells were *recA*$^-$ and *lexA*$^-$ (Basyuk *et al.*, 1994).

Microcin J.

Microcin J is a novel plasmid-encoded microcin produced by the uropathogenic *E. coli* isolate 2687. Cloning and mutagenesis has identified a 2.8kb stretch of DNA essential for MccJ production. Three complementation groups have been found and a possible 5 polypeptides identified by SDS-PAGE (Martin, 1994). DNA sequencing of this region has revealed at least three proteins with no apparent sequence similarity to known proteins. A further 2.2kb of sequence contiguous with the 2.8kb region has identified a 51kDa protein with sequence similarity to the MDR-like family of exporters. Transposon insertions into this region were found to render producing cells sensitive to MccJ (Kippenberger, 1996).

1.1.3 ECOLOGICAL ROLE OF COLICINS AND MICROCINS.

Escherichia coli is a ubiquitous organism in the intestine and is also a pathogen of several body systems, especially those involving mucosal surfaces. The role that colicins and microcins play in the ecology of *E. coli* is thought to be one of providing an advantage to the producing strain, thus defending the ecological niche against invading *E. coli* with the same nutrient requirements (Braun *et al.*, 1994). It has been suggested that colicins do not have a role in intestinal ecology due to the high activity of intestinal proteolytic enzymes (de Lorenzo and Aguilar, 1984). Colicins may however act outside the intestinal tract, for example in faeces, ensuring numerical dominance of the

producing strain resulting in a greater chance of faecal to oral transmission (Pugsley, 1984b). Microcins on the other hand may be of ecological significance due to their temperature and pH resistance (de Lorenzo and Aguilar, 1984). There is evidence to suggest that microcins are produced and remain active in the intestinal tract, since microcin-like compounds have been found in faecal samples (Baquero and Moreno, 1984).

Hardy (1975) identified three approaches to define the ecological role of colicins and microcins:(a) the use of gnotobiotic animals; (b) studies into bacterial succession; (c) comparisons between pathogenic and intestinal isolates.

Studies using gnotobiotic mice and pigs have both indicated that Col^+ bacteria predominate over Col^- bacteria, but it was found that the selective advantage was due to the Col factor and not through the killing of sensitive cells (Hardy, 1975).

The succession of intestinal bacteria has been studied in humans, mice and pigs. By dividing the bacterial species into resident strains, which survive in the intestine for several months, and transient strains, those retained for only a few days, it was found that resident bacteria were more likely to be Col^+ (Hardy, 1975). Experiments using mixed cultures of *Klebsiella pneumoniae* (MccE492) and intestinal *E. coli* have shown that the production and excretion of microcin results in a prevalence of the *Klebsiella pneumoniae*, since Mcc^- *Klebsiella* had no effect. The production of a microcin antagonistic substance indicated that the relationship between microcin production and predominance was not a simple one (de Lorenzo *et al.*, 1984). Studies into the intestinal flora of newborn infants have found that in five out of seven episodes of strain disappearance, the displaced strain was sensitive to the microcin produced by the new strain. In mice, *E. coli* containing pMccB17 were found to be 100 times more prevalent in faeces and lasted longer in the gut of the mice (Baquero and Moreno, 1984).

One of the best areas of evidence for the ecological significance of colicins and microcins is the high frequency of producing strains among natural populations (Pugsley, 1984b), and in particular the correlation between colicinogenicity and pathogenicity. Many studies have shown the high frequency of colicinogenicity among both pathogenic and non-pathogenic bacteria (Table 2). *E. coli* of human origin are more likely to be Col^+ than *E. coli* of animal origin (Riley and Gordon, 1992). Among pathogenic isolates an association between colicinogenicity and pathogenesis has been

determined (Smith, 1974; Smith and Huggins, 1976; Achtman *et al.*, 1983; O'Brien *et al.*, 1996). It has been shown that colicins remain active *in vivo* since activity was found in the blood of mice after subcutaneous injection with producing strains, and in the urine of mice suffering from a urinary tract infection (UTI) (Hardy, 1975). Among human UTI isolates, colicinogenicity was found to be correlated with symptomatic infections rather than asymptomatic infections (O'Brien *et al.*, 1996).

Table 2. Bacteriocin production among *E. coli* from pathogenic and non-pathogenic populations.

Study	Source[a]		% Col$^+$	% Mcc^{+b}
Smith, 1974	Bacteraemia:	Human	21.0	18.4[b]
		Bovine	25.0	41.7[b]
		Sheep	25.0	25.0[b]
		Chicken	15.4	66.7[b]
Asensio *et al.*, 1976	Human (H)		na	15.0
Baquero *et al.*, 1978	Human (H)		na	10.0
Davies *et al.*, 1981	Human:	Faeces	31.4	13.6[b]
	UTI	Urine(HO)	43.0	26.2[b]
	UTI	Urine(NH)	40.9	20.0[b]
	Septicaemia	Blood	51.0	31.6[b]
Waalwijk *et al.*, 1982	Uropathogenic		30.4	na
Achtman *et al.*, 1983	Human (D)		51.0	na
Riley and Gordon, 1992	ECOR collection		35	na
Khmel *et al.*, 1993	Human (H)		na	1.2
O'Brien *et al.*, 1996	Human: UTI		42.6	4.55

[a] Isolates from healthy (H) individuals or diseased (D) individuals. [b] Only colicinV tested for. Bacteriocins not tested for is denoted "na". Patients were hospitalised (HO) or non-hospitalised (NH). UTI: urinary tract infection isolate.

Colicin V and virulence.

To date only one *E. coli* bacteriocin, colicin V, has been directly associated with pathogenicity (Smith, 1974; Smith and Huggins, 1976; Davies, 1981; Achtman *et al.*, 1983). Conjugal transfer of the Col plasmid to avirulent strains was found to result in an increase in virulence, with loss of the plasmid returning the strain to its original state (Smith, 1974; Smith and Huggins, 1976). In order to determine if ColV itself caused the increase in virulence observed in many studies, Quakenbush and Falkow (1979) created Tn*5* mutants which abolished ColV production. The strains carrying the mutant plasmids still produced high mortality rates in mice, similar to those containing the wild type plasmids, suggesting that ColV itself does not play a role in virulence, but that other factors closely linked to the ColV genes in the plasmid may be responsible for the increase in virulence. More recently, it has been shown that ColV does have a direct role in virulence. Wooley *et al.* (1994) showed that avirulent strains transformed with the recombinant ColV plasmid pHK11, caused a significantly higher rate of mortality in chicken embryos compared to ColV$^-$ derivatives.

Colicin V associated virulence factors.

Several virulence factors have been found to be encoded by ColV plasmids (Fernandez-Beros *et al.*, 1990; Waters and Crosa, 1991). Adhesion, the specific binding of bacteria to epithelial cells at the site of attachment, is a prerequisite for colonisation (Reid and Sobel, 1987). Adherence to mouse intestinal epithelial cells has been found to increase when strains possess the plasmid pColV-H247 (Clancey and Savage, 1981), however this adhesion was not related to the expression of the F-pili (Darken and Savage., 1987). The conjugative pili have also been shown to increase cell surface hydrophobicity, the non-specific physiochemical attachment of bacteria to epithelial cell surfaces (Waters and Crosa, 1991).

Increased serum resistance is another property associated with ColV plasmids. Nilus and Savage (1984) identified three plasmids out of eight which encoded serum resistance. Resistance was associated with the plasmid transfer system but not solely dependent on plasmid transfer. A loci for serum resistance called *iss* (increased serum survival) has been cloned from pColV,I-K94 and was found to be closely linked to the

ColV genes (Binns *et al.*, 1979). Conjugative plasmids also encode a property called surface exclusion, in which the strains carrying the plasmid have a reduced ability to act as recipients if the donor harbours a closely related conjugative plasmid (Achtman *et al.*, 1977). One of the genes responsible for this property, *traT*, has been found to have a role in serum resistance (Moll *et al.*, 1980; Binns *et al.*, 1982). It has been suggested that TraT interferes with the formation of complement complexes, possibly at the junction sites between the inner and outer membrane (Binns *et al.*, 1982). Both the *traT* and *iss* genes are highly correlated with serum resistance and ColV production among clinical isolates (Montenegro *et al.*, 1985; Fernandez-Beros *et al.*, 1990).

The aerobactin-mediated iron assimilation system is one of the best recognised ColV associated virulence factors encoded on ColV plasmids (Warner *et al.*, 1981; Waters and Crosa, 1986). Williams (1979) was the first to identify the association, since ColV plasmids were found to confer a selective advantage to host bacteria in experimental infections which was reversible in conditions of excess iron. By the generation of ColV and aerobactin mutants and screening for the other activity, it was shown that iron uptake was independent of ColV synthesis (Williams and Warner, 1980). The aerobactin genes have also been identified in the chromosome of clinical isolates, but lack the conserved upstream region found in the plasmid encoded system (Valvano and Crosa, 1984; Bindereif and Neilands, 1985; Valvano *et al.*, 1986).

1.2 ABC TRANSPORTERS: STRUCTURE AND ROLE IN BACTERIOCIN TRANSPORT.

ABC transporters comprise a superfamily of transport proteins which utilise the energy from ATP hydrolysis to transport their specific substrate across the cell membrane. These transporters are found among prokaryotic organisms, both gram-positive and gram-negative bacteria and among eukaryotes (Higgins, 1992). Members of this family are defined by the possession of a highly conserved ATP-binding domain (Higgins *et al.*, 1986; Fath and Kolter, 1993).

A common feature of ABC transporters is the basic domain structure which is conserved in all examples found to date (Higgins *et al.*, 1986). Four domains have been identified of which two are hydrophobic membrane spanning domains (MSD) and two are cytoplasmic, containing the ATP-binding cassette (ABC). The transporter functions

either as a homodimer, with the ABC and MSD on the same polypeptide, or as a heterodimer where these domains reside on separate polypeptides (Higgins, 1992; Fath and Kolter, 1993). Phenotypic analysis has shown that the ABC superfamily has originated from a shared common ancestor, with the earliest divergence giving rise to two subfamilies, one comprised of homodimers and the other of heterodimers (Fath and Kolter, 1993).

Membrane Spanning Domain.

The MSD forms six transmembrane segments with three extracellular and two intracellular loops, resulting in each ABC transporter having twelve membrane spanning segments. The cytoplasmic loops are thought to interact with the ABC domain (Pearce *et al.*, 1992). Two transporters, MalF and HisQM, have been found to have only five transmembrane domains (Higgins, 1992).

ATP binding domain.

The ABC domain is approximately 200 amino acids long, located at the cytoplasmic C-terminus at the end of an α-helix. Two motifs, the A and B sites or Walker motifs, have been identified which form an ATP-binding pocket. Residues GXGKST are proposed to form a turn, bringing the lysine residue into close proximity to the phosphates in the Mg^{2+}-ATP. The aspartic acid in the B site is in close spatial proximity to the A site and may interact with the Mg^{2+} molecule (Walker *et al.*, 1982). The domain contains a highly folded core, with loops extending and interacting with other components of the transporter, coupling the energy from ATP hydrolysis to transport via conformational change. Loops 2 and 3 have been identified as the most likely to be involved in conformational change transduction, since mutations in these loops prevent transport without altering ATP binding and hydrolysis (Hyde *et al.*, 1990). A linker peptide, LSGGQ, which is central to the transmission of the ATP-dependent conformational change has also been found between the A and B sites (Koronakis *et al.*, 1995). By using the HylB transporter, the specific ABC transporter for α-hemolysin, Koronakis *et al.* (1995) have identified specific amino acids in the A, B and linker sequences which when substituted, disrupted energy translocation through loss of ATP hydrolysis but not binding. Substitutions K_{505} to I in the A site and D_{630} to H in the B site resulted in the loss of ATPase activity and the loss of export functions respectively, where as

substitutions S_{607} to N and G_{609} to D in the linker peptide resulted in the loss of export and ATPase activity but not ATP binding.

Types of ABC transporters.

ABC transporters have a high degree of specificity for their substrates which include sugars, peptides, inorganic ions, amino acids, oligopeptides, polysaccharides and proteins (Higgins, 1992). The superfamily can be divided into three families based on differences in sources and function (Reviewed by Fath and Kolter, 1993).

Bacterial importers.

Bacterial ABC importers, or periplasmic permeases, form a multisubunit import system, where the MSD and ABC domains reside on different polypeptides (Doige and Ames, 1993; Fath and Kolter, 1993). In prokaryotes, a periplasmic receptor protein is required to bind the substrate in the periplasm and present it to the import complex (Doige and Ames, 1993).

Eukaryotic transporters.

Eukaryotic ABC transporters have the ABC and MSD domains on the same polypeptide. There is a tandem repeat of the basic structure and no requirement for other subunits. Many eukaryotic transporters are of medical significance, for example the p-glycoprotein, responsible for multiple drug resistance in tumour cells when over expressed (Endicott and Ling, 1989) and the CFTR protein which is defective in cystic fibrosis patients (Riordan *et al.*, 1989).

Bacterial transporters.

The bacterial ABC transporters are involved in signal sequence independent export of substrates out of the cell. They have the basic domain structure previously described, with the ABC and MSD domains residing on the same or different polypeptides. Complementation between ABC transporters has been shown in several studies, with varying efficiency of export depending on the ABC transporter used (Guzzo *et al.*, 1991; Fath *et al.*, 1991; Binet and Wandersman, 1995). Discrimination of substrates has been found to be a function of the ABC transporter (Binet and Wandersman, 1995).

Accessory proteins have been identified in gram-negative bacteria which are required when the secreted product is destined for immediate release. These proteins have been defined as belonging to the membrane fusion protein family (MFP) and function to connect the inner and outer membranes (Dinh *et al.*, 1994). The genes encoding MFPs are linked to the ABC transporter genes (Fath and Kolter, 1993). With a few exceptions MFPs have a uniform size of 422 residues ±13% and have similar structures. The N-terminal region is hydrophilic, followed by 20 hydrophobic residues, forming an anchor in the inner membrane. Residues 150-318 are also hydrophilic, forming a central periplasmic domain. A hydrophobic C-terminal domain comprising residues 319-420 forms a highly conserved outer membrane domain. Phylogenetic grouping has been found to correlate with substrate specificity indicating a direct interaction between the MFP and substrate (Dinh *et al.*, 1994).

Outer membrane proteins have also been identified, the genes of which may be linked (Létoffé *et al.*, 1990) or distinct from the export genes (Wandersman and Delepelaire, 1990). A direct interaction between the MFP and outer membrane protein has been found, resulting in the direct passage of the substrate from the cytoplasm to the extracellular medium (Binet and Wandersman, 1995). Lipopolysaccharide biosynthesis is required for ABC mediated transport, possibly by ensuring the correct incorporation of outermembrane proteins in the cell envelope (Wandersman and Léttofé, 1993).

The substrates exported by bacterial ABC transporters can be divided into non-protein, protein and peptides (Fath and Kolter, 1993). Non-protein substrates include lipophilic drugs, antibiotics, and polysaccharides. No accessory proteins or outer membrane proteins are required since these products are not exported directly to the extracellular medium (Fath and Kolter, 1993).

The export signal of protein substrates resides in the C-terminal 60-150 residues, such as found with *E. coli* α-hemolysin (Kenny *et al.*, 1992) and *Erwinia chrysanthemi* proteases (Delepelaire and Wandersman, 1991). A glycine rich region, GGXGXD, close to the C-terminus is repeated six to nine times and may have a role in presenting the signal to the exporter (Wandersman, 1992).

Peptide transporters export ribosomally encoded bacteriocins produced by gram-positive and gram-negative bacteria. The export signal for these bacteriocins is located in the N-terminal 30 amino acids and shares a high degree of similarity. The term "double glycine leader peptide", has been given to these export signals since cleavage of the leader peptide occurs after two glycine residues (Håvarstein *et al.*, 1994). ABC exporters for peptide antibiotics have been found to have a N-terminal extension of 100-150 residues compared with other ABC transporters, which is responsible for the proteolytic cleavage of the leader sequence (Figure 8; Håvarstein *et al.*, 1995). The glycine residue at the -2 position appears to be central in the recognition and cleavage of the leader peptide (Gilson *et al.*, 1990; Håvarstein *et al.*, 1995). A model has been proposed in which the two proteolytic domains of the homodimer each bind a bacteriocin precursor. Hydrolysis of two ATP molecules results in a conformational change giving rise to cleavage and export of the bacteriocin (Håvarstein *et al.*, 1995).

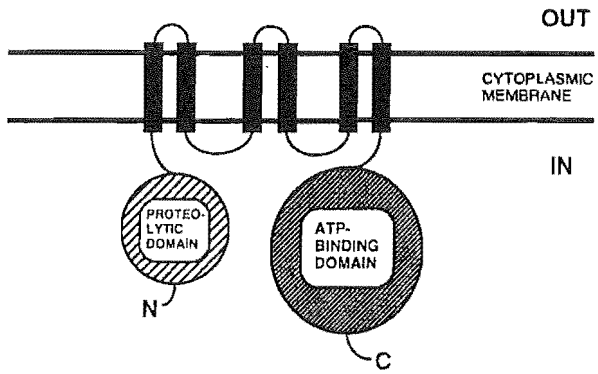


Figure 8. A model predicting the domain organisation of the new family of bacteriocin ABC transporters. Represented is an ABC transporter monomer, however these transporters probably function as homodimers. Consequently each transport complex contains two proteolytic domains, 12 membrane-spanning seqments and two ATP-binding domains (From Håvarstein *et al.*, 1995).

1.3 REGULATION OF BACTERIOCIN PRODUCTION.

1.3.1 SYNTHESIS DURING STATIONARY PHASE.

The synthesis of microcins is not inducible and seems to occur as the cells enter stationary phase (Kolter and Moreno, 1992). Starvation or stationary phase is defined as the phase when there is no detectable increase in cell numbers within a culture. This is a metabolically less active, more resistant state which allows the bacteria to survive in nutrient limiting environments and rapidly resume growth when nutrients are available (Reviewed by Seigele and Kolter, 1992; Kolter *et al.*, 1993).

Many global physiological and morphological changes occur during the entry of cells into stationary phase resulting in a variety of specific genes being transcribed. The promoter specificity of the RNA polymerase holoenzyme $\beta\beta'\alpha_2\sigma$ or $E\sigma$ (Helmann and Chamberlin, 1988) can be altered by replacing σ^{70}, the sigma factor required for gene expression during exponential growth, with alternative sigma factors in response to environmental signals. During the transition into stationary phase, 30 or more genes are expressed which are required for long term survival in a nutrient deficient medium (McCann *et al.*, 1991). The transcription of these genes is controlled by σ^S (σ^{38}) the stationary phase sigma factor, encoded by the *rpoS* gene, which has been previously known as *nur*, *appR*, *csi-2*, *abrD*, and *katF* (Loewen and Hengge-Aronis, 1994). Mulvey and Loewen (1989) first sequenced *rpoS* (*katF*) and determined an open reading frame of 1086 bp, with two potential transcription start sites and strong protein similarity to σ^{70}, suggesting that *rpoS* may function as a sigma factor. Loewen *et al.* (1993) identified the ATG at +37 as the transcriptional starting point resulting in a 342 amino acid protein (38 kDa). Further sequence comparisons between σ^{70}, σ^S and alternative sigma factors has identified a highly conserved region (region 2) involved in core binding, DNA melting and recognition of the -10 promoter sequence and a conserved C-terminal region (region 4) which recognises -35 promoter sequences (Lonetto *et al.*, 1992). Biochemical evidence that σ^S can function as a sigma factor binding RNA polymerase came from Nguyen *et al.* (1993) who were able to cosediment RNA polymerase and σ^S in a glycerol density gradient.

The recognition of different promoters by RNA polymerase is essential for the expression of genes required for survival in stationary phase. Both $E\sigma^{70}$ and $E\sigma^{S}$ are found to recognise very similar promoter sequences and in some cases can regulate the expression of the same gene (Tanaka *et al.*, 1993). The determination of a -10 and -35 consensus promoter sequence for σ^{S} binding has proved difficult (Loewen and Hengge-Aronis, 1994). A -10 hexamer, TATACT, has been determined for σ^{S}-dependent promoters, but can also be recognised by $E\sigma^{70}$ (Tanaka *et al.*, 1995), reflecting the similarity in their recognition domain, region 2.4 (Lonetto *et al.*, 1992). Unlike σ^{70} however, σ^{S} does not recognise a consensus -35 hexameric sequence, but requires additional sequences downstream of position -17 (Tanaka *et al.*, 1995). Some -35 promoter determinants are still required since changing the T to C found in the σ^{70}-dependent -35 hexamer of the *proU* promoter has been found to allow recognition by σ^{S}, whereas a change in the σ^{S} -dependent -35 promoter sequence of *osmY* from C to T resulted in recognition of the promoter by σ^{70} and decreased binding of σ^{S} (Wise *et al.*, 1996). DNA bending has been proposed as a mechanism to increase the strength of σ^{S}-dependent promoters, thus compensating for the absence of a well defined promoter sequence (Espinosa-Urgel and Tormo, 1993). Promoters containing "gearbox sequences" are another class of growth rate dependent promoters but are σ^{S}-independent (Vincente *et al.*, 1991). MccB17 expression is growth phase dependent, however transcription from P_{mcb}, which contains a gearbox sequence, is σ^{70}-dependent (Bohannon *et al.*, 1991).

The level of σ^{S} within the cell is tightly controlled and increases during entry to stationary phase to approximately 30% that of σ^{70} (Jishage and Ishihama, 1995). One promoter is responsible for the majority of *rpoS* transcription (Lange *et al.*, 1995) and corresponds to the P2 promoter determined by Takayanagi *et al.* (1994). Regulation of σ^{S} occurs at three levels, transcription, translation and protein stability (Lange and Hengge-Aronis, 1994), with transcription stimulated in response to 3'-5'-bispyrophosphate (ppGpp) (Gentry, 1993) and inhibited by cAMP-CRP (Lange and Hengge-Aronis 1991). During exponential growth, transcription of *rpoS* has been found to be high, however translation is induced dramatically during the transition to stationary phase implying post-transcriptional regulation of σ^{S} levels (McCann *et al.*,1993; Loewen *et al.*, 1993). The secondary structure of the *rpoS* mRNA has a long

and branched stem and loop structure in which the translation initiation sequence is paired with a downstream region preventing translation. Stabilisation of the mRNA, resulting in translation, requires the involvement of proteins specific for environmental signals (Lange and Hengge-Aronis, 1994). Protein stability is growth phase dependent and increases upon the onset of starvation. The ClpXP protease has been implicated in the instability of σ^S during exponential phase, however stability in stationary phase is not due to a decrease in ClpXP concentration suggesting another protein maybe involved (Schweder *et al.*, 1996). A response regulator encoded by the *rssB* gene, has been identified by Muffler *et al.* (1996) which is essential for σ^S turnover.

σ^S is part of a global regulatory system modulating gene expression in response to environmental signals such as temperature and osmolarity. Other factors such as cAMP-CRP, Lrp, IHF and H-NS act independently or in combinations to regulate σ^S-dependent genes, fine tuning gene expression (Lange *et al.*, 1993; Loewen and Hengge-Aronis, 1994). Virulence has also been found to be σ^S regulated. The plasmid encoded *spvRABCD* genes are highly conserved among non-typhoidal *Salmonella* serovars and are required for survival within phagosomes (Fang *et al.*, 1992; Guiney *et al.*, 1995). Mutations in *rpoS* reduce the transcription of *spvR*, the regulator of *spvABCD*, resulting in reduced virulence. Complementation using cloned *rpoS* restored virulence through increasing *spvR* transcription. It was also thought that *rpoS* also regulates chromosomal genes required for virulence (Kowarz *et al.*, 1994).

1.3.2 HISTONE-LIKE PROTEINS.

The histone-like nucleoid structural protein (H-NS or H1) is a heat stable, neutral protein which is highly conserved in enterobacterial species and functions as a homodimer in binding double stranded DNA. Along with the protein HU, H-NS forms the structural component of the bacterial nucleoid (Reviewed by Drlica and Rouviere-Yaniv, 1987; Higgins *et al.*, 1990; Ussery *et al.*, 1994). The gene encoding H-NS, *hns*, is chromosomally encoded and has been identified as *osmZ*, *bglY*, *drdX*, *pilG* and *virR* in several studies (Ussery *et al.*, 1994). A single copy of *hns* was mapped to 27 minutes on the *E. coli* chromosome (Pon *et al.*, 1988). Mutations in *hns* are found to be highly pleiotrophic, affecting the expression of apparently unrelated plasmid and chromosomal genes, indicating that H-NS is involved in gene regulation (Higgins *et al.*, 1990).

Spassky *et al.* (1984) identified species of H-NS, a, b and c which exist in varying ratios depending on growth conditions. The level of *hns* transcription is maximal at mid-logarithmic phase and no significant accumulation of H-NS occurs during stationary phase (Free and Dorman, 1995).

Many genes are regulated by H-NS, including the cold shock regulon (La Teana *et al.*, 1991) and osmoregulated genes (Higgins *et al.*, 1988), as well as virulence genes in *Shigella flexneri* and *E. coli* (Dorman *et al.*, 1990; Tobe *et al.*, 1993; Colonna *et al.*, 1995) and ribosomal RNA synthesis (Tippner *et al.*, 1994). H-NS alters gene expression by functing as a regulator or as a passive structural component of the nucleoid (Ussery *et al.*, 1994), inducing changes in the topology of DNA (Hulton *et al.*, 1990). Yamada *et al.* (1991) were the first to identify H-NS as a *trans* acting transcriptional regulator by analysing H-NS deletion mutants by 2D-gel electrophoresis and finding the induction of at least 21 genes. Further studies by Ueguchi and Mizuno (1993) using the *proU* promoter identified H-NS as a transcriptional repressor, binding to target promoters and inhibiting an early step(s) of transcription initiation by a direct mechanism rather than through alteration of the supercoiled state of the promoter. The cAMP-CRP complex has been found to act as an antirepressor for H-NS-dependent transcriptional repression (Forsman *et al.*, 1992). Although H-NS acts primarily as a transcriptional repressor, in some instances however, the level of some proteins decreases in an *hns* mutant implying that H-NS is functioning as a transcriptional activator (Yamada *et al.*, 1991). The precise mechanism of transcriptional activation has yet to be identified (Ussery *et al.*, 1994).

DNA bending has been shown to be a mechanism which affects gene transcription through changing or determining the affinity of regulatory proteins, whether activators or repressors, to cognate sites in the neighbourhood of the RNA polymerase binding site (Pérez-Martín *et al.*, 1994). The sites of DNA curvature most often occur in AT rich regions upstream or down stream of the transcription initiation site (Travers and Klug, 1987; Crothers *et al.*, 1990). Owen-Hughs *et al.* (1992) identified sequences downstream of the *proU* promoter as the site of H-NS contact. This region of curved DNA could be replaced by heterologous curved DNA, indicating that H-NS recognises and binds to a structural motif, thereby preventing RNA polymerase binding and hence transcription initiation. H-NS binding sites have been

shown to correspond with RNA polymerase and CRP binding sites (Rimsky and Spassky, 1990). By placing a 5A6A motif in front of the *lacZ* gene, Zuber *et al.* (1994) were also able to show that H-NS binds to curved DNA sequences and represses transcription. The *hns* promoter also contains a region of curved DNA which binds H-NS resulting in autoregulation of *hns* transcription (Ueguchi *et al.*, 1993; Falconi *et al.*, 1993). Autoregulation controls H-NS concentration in response to growth rate therefore adjusting the nucleoid in response to environmental conditions (Dersch *et al.*, 1993). Blockage of DNA synthesis has been found to shut-down *hns* transcription, therefore autoregulation also matches H-NS synthesis to the demands of DNA synthesis, maintaining a relatively constant H-NS-DNA ratio (Free and Dorman, 1995).

Recently H-NS has been identified as a component of the *rpoS* global network, with at least 21 σ^S-dependent genes derepressed in an *hns*$^-$ background (Barth *et al.*, 1995). Three classes of σ^S-dependent genes have been determined with respect to the effect of H-NS (Barth *et al.*, 1995). Class I genes, typified by *osmY*, are no longer affected by *hns*$^-$ mutations in an *rpoS*$^-$ background, suggesting that H-NS has an indirect effect on gene expression through regulation of *rpoS*. Yamashino *et al.* (1995) have shown that H-NS is involved in post-transcriptional regulation of σ^S. In *hns::neo* mutants there was a 10 fold increase of σ^S at mid-logarithmic phase. This increase was not due to increased transcription of *rpoS*, but rather due to increased translational efficiency of *rpoS* mRNA and σ^S stability. It was thought that H-NS affects the translation efficiency through negatively regulating a gene which encodes a factor involved in *rpoS* mRNA translation or by directly binding the mRNA. Increased stability of σ^S could occur through H-NS repressing the transcription of a gene encoding a factor responsible for σ^S stability. Class II genes, for example the MccC7 genes (Moreno *et al.*, 1992), are more strongly expressed in *hns*$^-$ *rpoS*$^-$ backgrounds than in *rpoS*$^-$ alone. It is possible that H-NS can bind these genes directly. In the *hns*$^-$ *rpoS*$^-$ background, gene expression is modulated by σ^{70} however, in a wild type strain, only σ^S can overcome H-NS repression. Expression from the MccB17 promoter P_{mcb} is also increased in a *bglY* (*hns*) mutants (Moreno *et al.*, 1992), however transcription is σ^{70} dependent (Bohannon *et al.*, 1991). Class III genes , for example *csiD* and *csiE*, are weakly increased in *hns* mutants during exponential growth and seem to require H-NS as a positive factor for stationary phase growth as well as an additional factor besides

σ^S. Expression from the MccB17 promoter P_{mcb} would appear to fall into this category since transcription is increased in *bglY* (*hns*) mutants and requires *ompR* as a positive activator (Moreno *et al.*, 1992) however, transcription is σ^{70} dependent (Bohannon *et al.*, 1991).

Other DNA-binding proteins.

Alternative histone-like proteins have been identified in *E. coli and Yersinia enterocolitica*. StpA is a second H-NS-like protein in *E. coli* which appears to be a molecular backup for H-NS. StpA also regulates gene expression independently of H-NS, possibly through superior binding to RNA (Zhang and Belfort, 1992; Zhang *et al.*, 1996). The *ymoA* gene of *Y. enterocolitica*, encodes a histone-like protein which modulates expression of thermoregulated virulence factors. The predicted protein sequence encoded by *ymoA* however has no significant similarity to other known histone-like proteins (Cornelis *et al.*, 1991).

Two other histone-like proteins are also found in *E. coli*, but unlike HU and H-NS which bind DNA non-specifically, IHF and FIS both bind DNA in a site-specific fashion (Reviewed by Drlica and Rouviere-Yaniv, 1987; Schmid, 1990; Freundlich *et al.*, 1992; Goosen and van de Putle, 1995). The integration host factor, IHF, was first identified as being involved in the site specific recombination of phage λ. Now the involvement of IHF in a wide variety of physiological activities (Friedman, 1988) and transcriptional regulation, including transcription from the MccB17 promoter P_{mcb} (Moreno *et al.*, 1992) is well recognised. IHF is a 21.8 kDa protein encoded by *himA* and *himD* and functions as a heterodimer, binding approximately 40 bp of DNA around a 13 bp consensus sequence resulting in DNA bending (Goosen and van de Putle, 1995). The consensus sequence is located within the AT rich promoter regions of IHF regulated promoters (Goodrich *et al.*, 1990; Harrington, 1992). Transcription from *himA* and *himD* increases as the cells enter stationary phase and is controlled by σ^S, ppGpp and IHF (Aviv *et al.*, 1994). IHF affects transcription through both passive and active mechanisms by being a component of the bacterial nucleoid or through acting as a positive or negative regulator of gene expression (Goosen and van de Putle, 1995). Because IHF binds bent DNA, it is postulated that H-NS and IHF might alter DNA

supercoiling resulting in fine tuning of gene expression (Goosen and van de Putle, 1995).

1.3.3 IRON REGULATION.

The ferric uptake regulation protein, Fur, is the central regulator of gene expression for all genes involved in iron uptake in *E. coli* (reviewed by Bagg and Neilands, 1987, Silver and Walderhaug, 1992; Guerinot, 1994). Other genes not related to iron metabolism are also Fur regulated including carbon utilisation pathways (Bagg and Neilands, 1987) and virulence gene expression (reviewed by Litwin and Calderwood, 1993) such as Shiga-like toxin production (Calderwood and Mekalanos, 1987 and 1988) and ColV production and uptake (Chehade and Braun, 1988). Fur homologs have been identified in other gram-negative bacteria including pathogens, with greater than 50% identity found between Fur proteins at the amino acid level (Ochsner *et al.*, 1995).

The Fur protein, encoded by *fur*, is a 148 amino acid protein which functions as a transcriptional repressor, requiring activation by a divalent heavy metal ion. *In vivo* Fur binds to Fe^{2+}, however other divalent ions such as Mn^{2+}, Co^{2+} Cd^{2+} and Zn^{2+} have also been shown to bind Fur (Bagg and Neilands, 1987). The histidine residues 31, 32 and 131 have been implicated in ion binding (Saito *et al.*, 1991). Fur regulated repression also functions as part of the global regulatory network, fine tuning gene expression to environmental signals, as seen in *E. coli* manganese superoxide dismutase expression, where two positive and four negative regulators function to control gene expression (Compan and Touati, 1993).

Functioning as a dimer, Fur represses transcription through binding to a specific sequence located within the promoters of Fur regulated genes including *fur* itself. The "Fur-box", first identified in the aerobactin operon (de Lorenzo *et al.*, 1987; Bagg and Neilands, 1987), is a 19bp dyad repeat containing the sequence 5'-TCATT-3' as a complement in both halves of the repeat (Calderwood and Mekalanos, 1988). Visualisation of the Fur-DNA interaction indicates that Fur wraps around the DNA fragment, partially or totally covering it, resulting in rigidification of the DNA and

polymerisation of Fur (Le Cam *et al.*, 1994), therefore preventing transcription by inhibiting RNA polymerase binding. Repression is relieved in iron limiting environments since Fur alone cannot bind DNA without the presence of a divalent ion, and so Fur dissociates from the promoter allowing RNA polymerase to bind (Litwin and Calderwood, 1993).

1.4 MICROCIN 24.

Microcin 24 is produced by a uropathogenic *E. coli* strain, 2424, isolated from a patient with pyelonephritis at Christchurch Hospital. Previously classified as colicin 24, this bacteriocin was found to be novel by using cross-immunity tests against the colicin reference collection (Pugsley and Oudega, 1987). Activity was found against all of the colicin or microcin producing strains as well as against *Salmonella typhimurium* (O'Brien and Mahanty, 1994).

The genes encoding Mcc24 reside on a 43.54 kb plasmid, p24-2. Mutagenesis has identified two consecutive *EcoRI* fragments that contain the Mcc24 genes (Figure 9). Failure to restore a $Mcc24^+$ phenotype in mutant strains by lysing with chloroform vapour, a technique previously used to identify transport mutants in the study of ColV (Gilson *et al.*, 1987), did not allow complementation groups to be determined. In order to clone the Mcc24 genes, an extra *Bam*HI site within mini-Tn*10* was utilised from a mutant with a $Mcc24^+$ phenotype (Figure 9, no 24). The resulting pBR322 clone, pGOB34, contained 25.14 kb of insert DNA (O'Brien and Mahanty, 1994).

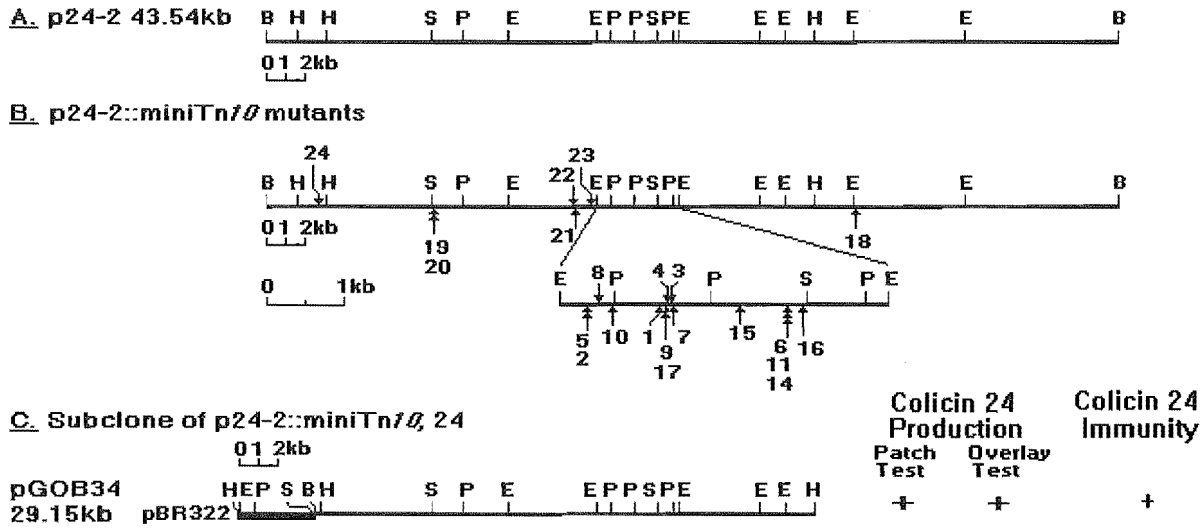


Figure 9. Restriction map of p24-2, mini-Tn*10* mutants and pGOB34. (From O'Brien and Mahanty, 1994) **A.** Restriction map of p24-2. Restriction enzymes B, *Bam*HI; E, *Eco*RI; H *Hin*dIII; P, *Pst*I; S, *Sal*I. **B.** Location of mini-Tn*10* insertions in plasmid p24-2 resulting in various Mcc24 phenotypes. Microcin phenotypes: normal, 24; increased, 18-20; reduced 21-23; minus, 1-11 and 14-17. **C.** Restriction map of pGOB34. Cloning utilised a unique *BamHI* site in mutant 24 present in mini-Tn*10* resulting in a 25.14kb fragment being cloned into pBR322.

1.5 AIMS OF THIS STUDY.

Colicins and microcins are the two classes of bacteriocins produced by *E. coli* and are often produced by pathogenic *E. coli* strains, however the specific role that these toxins play in pathogenicity is unclear. Of the colicins and microcins studied to date, the majority are produced by strains isolated in Europe and the USA with only three microcins, ColV, MccB17 and MccC7 having been studied in detail.

Because of the unique nature of Mcc24 and the strong bacteriocidal activity this toxin displayed against other enteric pathogens, this study aimed to elucidate the molecular mechanisms of Mcc24 production, immunity, transport and activity in order to further understand the role that microcins play in bacterial pathogenicity. The specific objectives of this study were:

1. To subclone the Mcc24 encoding genetic region to the smallest possible DNA fragment which retained all of the Mcc24 activity and regulation.
2. Identify the number of genes involved in Mcc24 production, immunity and transport through mutagenesis and complementation, utilising *lacZ* fusions to determine the number and location of the promoters regulating transcription of the Mcc24 genes.
3. Obtain the complete nucleotide sequence of the Mcc24 encoding region and confirm the open reading frames through mutagenesis, complementation and protein analysis using *E. coli* maxicells or minicells.
4. Identify mechanisms of Mcc24 regulation.
5. Extract Mcc24 from spent culture supernatants in order to elucidate the mechanism of action against target cells.
6. Test the activity of Mcc24 against known human pathogens and assay microcin producing clones for toxicity using an animal model.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 BACTERIAL STRAINS, BACTERIOPHAGES AND PLASMIDS.

Bacterial strains, bacteriophages and plasmids used in this study are listed in Table 4.

2.2 BUFFERS AND MEDIA.

Buffers and solutions used in this study were prepared as described in Appendix 1.

Media used in this study were prepared as described in Appendix 2.

2.2.1 ANTIBIOTICS AND SUPPLEMENTS.

Antibiotics and supplements were added to agar plates or to overnight liquid cultures as stated in Table 3.

Table 3. Antibiotics and Supplements.

Antibiotic or supplement	Abbreviation	Concentration
Ampicillin	Amp	50 or 100mg/ml
Chloramphenicol	Cm	30mg/ml
Gentamycin	Gent	30mg/ml
Kanamycin	Kan	50mg/ml
Nalidixic acid	Nal	30mg/ml
Rifampicin	Rif	50 - 150mg/ml
Spectinomycin	Spec	50mg/ml
Streptomycin	Strep	50mg/ml
Tetracycline	Tet	15mg/ml
Isopropyl-β-D-thio-galactopyranoside	IPTG	0.5mM
5-bromo-4-chloro-3- indoyl-β-D-galactopyranoside	X-Gal	30mg/ml
o-Nitrophenyl-β-D-galactopyranoside	ONPG	0.8mg/ml
Sodium pyrophosphate	Napp	1.25mM
2,2'-dipyridyl		0.1mM

Table 4. Bacterial Strains, Bacteriophages and Plasmids.

Strain, Phage or Plasmid	Genotype or description	Reference
Escherichia coli		
BzB1013	*Fep* mutation *FepA* Resistance-ColB ColD	Pugsley and Oudega, 1987
BzB1030	*butB* mutation Resistance-ColA ColE1 ColE2-E9	Pugsley and Oudega, 1987
BzB1190	*tsx* mutation Resistance-ColK	Pugsley and Oudega, 1987
BzB1192	*tonB*$^-$	Pugsley and Oudega, 1987
BzB2101	(pColA-CA31 ColA$^+$ *caa cai cal*)	Pugsley and Oudega, 1987
BzB2104	(pColE1-K53 ColE1$^+$ *ceaA ceiA celA*)	Pugsley and Oudega, 1987
CSH26	pColV-K30::Tn*10* Tet^R ColV$^+$	*
DH10B	F$^-$ *araD*139 Δ(*ara, leu*)7697 Δ*lacX*74 *galU galK mcrA* Δ(*mrr-hsdRMS-mcrBC*) *rpsL dor* ϕ80d*lacZ*ΔM15 *endA*1 *nupG recA*1	Grant *et al.*, 1990
DF1	*rspl* (*tnp*A Cm^R)	Wang *et al.*, 1993a; 1993b
GC4468	F$^-$ Δ*lacU*169 *rpsl fur*$^+$.	Compan and Touati, 1993
GC4468	*fur*$^+$ (pColV-K30::Tn*10* Tet^R ColV$^+$)	This study
GC4468	*fur*$^+$ (p24-2::mini-Tn*10* # Mcc24$^+$)	This study
GC4468	Δ*fur*::*kan*	Compan and Touati, 1993
GC4468	Δ*fur*::*kan* (pColV-K30::Tn*10* Tet^R ColV$^+$)	This study
GC4468	Δ*fur*::*kan* (p24-2::mini-Tn*10* # Mcc24$^+$)	This study
GC7442	F$^-$ *his trpE tolC*::Tn*5* ColV leaky	Gilson *et al.*, 1990
GC7459	*tolC*::Tn10 ColV tight	Gilson *et al.*, 1990
JM246	*cys*-	*
JM246	*cys*- Mcc24^R	This study
K802	*met*-	*
K802	*met*- Mcc24^R	This study
L392 (ZK127)	*supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 *lacY*1	Murray *et al.*,1977
LE392	*metB*1 Mcc24^R	This study
MC4100	F$^-$ *araD*139 Δ(*lacI PO ZYA-argF*) U169 *rpsL thi recA*-56	Casadaban, 1976
MC4100	Mcc24^R	This study
MC4100	*recA*-56 (pHK11 ColV$^+$ Amp^R)	Gilson *et al.*, 1987
N3002	*cys*-	*
N3002	*cys*- Mcc24^R	This study

Table 4. Continued.

Strain, Phage or Plasmid	Genotype or description	Reference
Escherichia coli		
PAP308	*ompF* mutation Resistance-ColA ColN Tolerance-ColE2-E9 ColK ColL ColS4 mccC7 mccB17	Pugsley and Oudega, 1987
PAP702	*ompA* mutation Tolerance-ColK ColL	Pugsley and Oudega, 1987
PAP710	*semA*::Tn*5* Tolerance-E492	Pugsley and Oudega, 1987
PAP1402	*ompR*::Tn*5* Resistance-ColA CoLN Tolerance-ColE2-E9 ColK ColL ColS4 ColB18	Pugsley and Oudega, 1987
PB2480	F^- *thr*1 *leu*6 *lacY*1 *supE*44 *tonAZ*1 T_SR 080^r	Berquist,P.
P678-54T	F^- *thr leu thi supE lacY tonA gal mal xyl ara mtl min*	Meagher *et al.*, 1977
W3110	*E.coli* K-12 F^- lambda$^-$ $Strep^R$	*
W3110	(p24-2::mini-Tn*10* Kan^R #24 $Mcc24^+$) $Strep^R$	O'Brien, 1992
ZB3	ZK126 *rpoS*(IS insert) λ(bolA::lacZ) lysogen Kan^R	*
ZK126 (ZK353)	*sup*$^-$ $Strep^s$ F^- Rif^r	Kolter, R.
ZK126	(pJC10 $MccJ^+$)	Martin, 1994
ZK126	(pColV-K30::mini-Tn*10* Tet^R $ColV^+$)	This study
ZK126	(pHK11 $ColV^+$ Amp^R)	This study
ZK762	MC4100 *pcnB80 zad*::Tn*10*	Lopilato *et al.*, 1986
71-18	*supE thi* Δ(*lac-proAB*) F' [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Messing *et al.*, 1977
2424	(p24-12 Amp^R Cm^R $Strep^R$ Tet^R $Spec^R$ $Mcc24^+$) Nal^R Hospital pyelonephritis isolate	O'Brien and Mahanty, 1994
2424	(p24-2 $Mcc24^+$) Nal^R	O'Brien and Mahanty, 1994
2686	$MccJ^+$ Hospital pyelonephritis isolate	Martin, 1994
Other		
K. pneumoniae	RYC472 $MccE492^+$	Pugsley and Oudega, 1987
S. typhimurium LT2		*
Hospital pathogens	Pathogen reference strains. Table 9.	Christchurch Hospital
Bacteriophage		
Lambda NK1316	1.8 kb mini-Tn*10* 103 Kan^R	Kleckner *et al.*, 1991
Lambda NK1323	2.9 kb mini-Tn*10* 104 Tet^R	Kleckner *et al.*, 1991
Lambda NK1205	4.9 kb mini-Tn*10* 112 Kan^R *lacZ*	Kleckner *et al.*, 1991

Table 4. Continued.

Strain, Phage or plasmid	Genotype or description	Reference
Plasmid		
pACYC184	Cm^R Tet^R	Chang and Cohen, 1978
pBluescript	SK+ Amp^R	Short *et al.*, 1988
pBR322	Amp^R Tet^R	Bolivar *et al.*, 1977
pColV-K30::Tn*10*	$ColV^+$ Tet^R	Gilson et al., 1987
pDELTA1	Amp^R Kan^R Tet^R *strA sacB lacZ'*	Wang *et al.*, 1993a; 1993b
pGOB34	Amp^R $Mcc24^+$	O'Brien and Mahanty, 1994
pGOB9	6.12kb *Sau*3AI fragment in pBR322, Amp^R $Mcc24^+$	This study
pGOB11	6.72kb *Sau*3AI fragment in pBR322, Amp^R $Mcc24^+$	This study
pGOB18	5.25kb *Sau*3AI fragment in pBR322, Amp^R $Mcc24^+$	This study
pGOB18::mini-Tn*10*	Insertions with various Mcc24 phenotypes, Kan^R	This study
pGOB181	1.913kb *Eco*RI fragment in pBR322, Amp^R $Mcc24^-$	This study
pGOB181::mini-Tn*10*	Insertions with various Mcc24 phenotypes, Tet^R	This study
pGOB200	7.65kb *Eco*RI/*Sal*I fragment in pUC18, Amp^R $Mcc24^-$	This study
pGOB341	9.3kb *Bam*HI/*Eco*RI fragment in pBR322, Amp^R $Mcc24^-$	This study
pGOB342	4.5kb and 4.2kb *Eco*RI fragments in pUC18, Amp^R $Mcc24^+$	This study
pGOB343	7.86 kb *Sal*I/*Hin*dIII fragment in pBR322, Amp^R $Mcc24^-$	This study
pGOB420	4.5kb and 4.2kb *Eco*RI fragments in pUC18, Amp^R $Mcc24^+$	This study
pGOB421	4.2kb *Eco*RI fragment in pUC18, Amp^R $Mcc24^-$	This study
pGOB423	4.5kb *Eco*RI fragment in pUC18, Amp^R $Mcc24^-$	This study
pGΔ185	5.267kb insert + 3.99kb (pBR322) *Bam*HI/*Eco*RI fragment in pDELTA1, Amp^R Kan^R Tet^R $Mcc24^+$	This study

Table 4. Continued.

Strain, Phage or plasmid	Genotype or description	Reference
pGΔ185	Deletion derivatives, 33 Kan^R/$Strep^R$, various phenotypes	This study
pGΔ813	1.913kb insert + 0.28kb pBR322 *Eco*RI/*Sal*I fragment in pDELTA1, Amp^R Kan^R Tet^R $Mcc24^-$	This study
pGΔ813	Deletion derivatives, 19 Tet^R/Suc^R and 11 Kan^R/$Strep^R$, various phenotypes	This study
pGΔ851	3.36kb *Eco*RI fragment in pDELTA1, Amp^R Kan^R Tet^R $Mcc24^-$	This study
pGΔ851	Deletion derivatives, 14 Tet^R/Suc^R and 14 Kan^R/$Strep^R$, various phenotypes	This study
pGSK813	1.913kb insert + 0.28 kb pBR322 *Eco*RI/*Sal*I fragment in pBluescript, Amp^R $Mcc24^-$	This study
pHK11	*cvaC*$^+$ *cvi*$^+$ *cvaA*$^+$ *cvaB*$^+$ $ColV^+$, Amp^R	Gilson *et al.*,1987
pHK22	*cvaC*$^+$ *cvi*$^+$ *cvaA*$^+$ *cvaB*$^+$ $ColV^+$,Cm^R	Gilson *et al.*, 1987
pHK22::Tn*5* #6	$ColV^-$ Cm^R Kan^R	Gilson *et al.*, 1987
pLOB8	3.7kb *Sau*3AI fragment in pACYC184, Cm^R $Mcc24^-$	This study
pLOB21	1.3kb *Sau*3AI fragment in pACYC184, Cm^R $Mcc24^-$	This study
pLOB421	4.2kb *Eco*RI fragment in pACYC184, Cm^R $Mcc24^-$	This study
pLOB813	1.913kb *Bam*HI fragment in pACYC184, Cm^R $Mcc24^-$	This study
pLOB813::mini-Tn*10*	Insertions with various Mcc24 phenotypes, Tet^R	This study
pLY21	*cva*C$^+$ *cvi*$^+$ *cvaAB*$^-$ Cm^R	Gilson *et al.*, 1990
pNK2882	*tnpA* ATS transposase Tet^R	Kleckner *et al.*, 1991
pUC18	Amp^R	Yanisch-Perron *et al.*, 1985
pYIE10		Shum, 1992
p24-2	$Mcc24^+$	O'Brien and Mahanty, 1994

* = Laboratory Collection.

2.3. BACTERIOLOGICAL METHODS.

2.3.1 CULTURE CONDITIONS.

Bacterial cultures were incubated at 37°C unless otherwise stated. Strains used in the experiments outlined in Results 3.6.1 were incubated at the appropriate temperature of each organism.

Exponential Cultures.

A 1:50 dilution of a bacterial overnight culture was made in LB broth (50-100ml). The culture was vigorously shaken at the required temperature for 2-3 hours until the culture reached an OD_{600} of 0.4.

Overnight Cultures.

A single bacterial colony was resuspended in 3ml LB broth containing antibiotics when required and shaken at the appropriate temperature overnight. When required the cells were pelleted by centrifugation (6000rpm, 5min, 4°C). An overnight culture was assumed to contain approximately $1x10^9$ cells/ml (Kleckner *et al.*, 1991).

2.3.2 ANTIBIOTIC RESISTANCE.

Plate assay.

Bacterial strains were subcultured on the appropriate antibiotic plate in order produce single colonies. Discrete colonies on the second and third streaks denoted resistance.

Serial dilution.

A loopful of bacteria was resuspended in 100µl of LB in a microtitre well. Serial dilutions of 10^{-2}, 10^{-4} and 10^{-6} were made in LB broth to a final volume of 100µl. A 5µl sample from each dilution was spotted onto the appropriate antibiotic plates. After drying, the plates were incubated at overnight and growth at 10^{-2}, 10^{-4} or 10^{-6} was taken to denote antibiotic resistance.

2.3.3 COLICIN AND MICROCIN PRODUCTION (Pugsley and Oudega, 1987).

Plate overlays were performed using a 3ml aliquot of molten H-top agar inoculated with an overnight culture of *E. coli* MC4100 (100µl) unless otherwise stated.

Patch Test.

Post-incubation.

A M63 plate was overlayed and after 20 minutes, the test strains were toothpicked onto the lawn and the plates incubated for 8-14 hours. Bacteriocin production was scored visually, based on the distance between the colony edge and the edge of the clearing zone (1=<0.5mm; 2=0.5-1mm; 3=1-2.5mm; 4=2.5-5mm; 5=>5mm).

Pre-incubation.

In some instances it was necessary to pre-incubate the test strains after spreading 100µl of LB broth over a M63 plate to provide a limited nutrient source. The test strains were then toothpicked onto the plate. After overnight incubation at 30°C, the plate was overlayed with H-top agar seeded with *E. coli* MC4100 and incubated for 8-14 hours.

Overlay Test.

The test colonies were toothpicked onto a M63 plate and grown as described for the patch test with pre-incubation. The plate was exposed to chloroform vapour for 20 minutes and then left open to allow the residual chloroform to evaporate. A 5ml aliquot of molten H-top agar seeded with indicator bacteria was used for the overlay and the plate incubated as previously described.

Cross-streak Test.

Using a sterile toothpick or loop, a bacteriocin-producing strain was streaked down the centre of a M63 plate and incubated for 48 hours. The test strains were streaked at right angles up to the edge of the producing strain streak and incubated overnight. Inhibition of growth near the junction between producing and test strains denoted sensitivity of the test strain to the bacteriocin.

Cellophane test.

Pre-incubation.

Cellophane filters were cut to fit into a petri dish and sterilised under UV light for 5 minutes on each surface. A filter was placed on a M63 plate and the test strains toothpicked onto the surface of the cellophane and incubated overnight. The following day the filter was removed and 5ml of molten H-top agar seeded with indicator bacteria was poured onto the plate and the plate incubated for 8 hours.

Post-incubation.

The test strains were grown as previously described for the patch test with pre-incubation. A cellophane filter was placed over the colonies and 3ml of molten H-Top agar seeded with indicator bacteria gently was poured over the plate, followed by an 8 hour incubation.

2.3.4 GENERATION OF SPONTANEOUS MICROCIN 24 RESISTANT MUTANTS.

The strain from which spontaneous mutants were to be derived was used to seed 3ml H-Top agar and poured over a M63 agar plate. Colonies of MC4100(pGOB18) were spotted onto the lawn and the plate incubated for 12-24 hours. Any colonies that appeared within the microcin halo surrounding MC4100(pGOB18) were collected, purified and retested for microcin resistance using the patch test.

Amino acid uptake.

Overnight cultures of two $Mcc24^R$ mutants were grown in LB broth and a loopful streaked for single colonies on M63 agar and M63 agar supplemented with methionine (20μg/ml) or cysteine (20μg/ml). Modification to this method involved harvesting the cells from 1ml of overnight culture, washing the cells in 1x M63 salts and resuspending the pellet in 1ml of 1x M63 salts. Serial dilutions of 10^{-2}, 10^{-4} and 10^{-5} were made and a 10μl aliquot from each was plated on a M63 agar and M63 agar with amino acid supplements. After an overnight incubation the plates were examined for growth.

2.4 DNA MANIPULATION AND CLONING TECHNIQUES.

All centrifugation steps involving DNA preparation were performed at 12000rpm for 5 minutes at 4°C unless otherwise stated. When required, enzymes were inactivated by heating at 75°C for 10 minutes.

2.4.1 ALKALINE EXTRACTION OF PLASMID DNA.

(Derived from Birnboim and Doly, 1979).

A 1.5ml aliquot of bacterial overnight culture was poured into an eppendorf tube and the cells pelleted by centrifugation. The supernatant was removed by suction and the pellet resuspended in 100µl of Solution I. After a five minute incubation on ice, 200µl of Solution II was added and mixed gently until the solution became viscous and clear. After a further 10 minute incubation on ice, 150µl of Solution III was added and the tube mixed thoroughly to break up the precipitate. The tube was incubated on ice for one hour, the precipitate pelleted by centrifugation and the supernatant poured off into a new tube. Sometimes a second centrifugation was necessary to obtain a clean lysate.

The DNA was precipitated using 250µl of cold isopropanol and placed on ice for 30 minutes. Once pelleted, washed with 1ml of 70% ethanol and air dried, the DNA was dissolved in 200µl of 0.3M sodium acetate (pH 4.8), and 400µl of cold 100% ethanol added. The DNA was precipitated at -80°C for 10 minutes and pelleted by centrifugation. Aspriration was used to remove the supernatant and a second spin in a microfuge performed to remove any remaining liquid, after which the DNA pellet was dissolved in 30µl TE.

2.4.2 MODIFIED ALKALINE EXTRACTION OF PLASMID DNA

(Kennedy, 1988. A modified version of Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981).

The same solutions and volumes as in the above procedure were used. After Solutions I and II the tube was incubated at room temperature for five minutes. Following the addition of Solution III, the tube was placed on ice for a further five minutes and the precipitate pelleted by centrifugation. The supernatant was transferred to a new tube and 1ml of room temperature 100% ethanol was added. After two minutes at room

temperature, the DNA was pelleted by centrifugation (12,000rpm, 10min, 25°C), the supernatant removed by aspiration and the pellet washed with 400µl room temperature 70% ethanol. Once dried, the pellet was dissolved in 30-50µl of sterile distilled water.

2.4.3 LARGE SCALE PLASMID DNA PREPARATION - Lithium Chloride Method (Sambrook et al., *1989).*

Bacterial cultures were grown overnight in 100ml of LB broth with the appropriate antibiotics and then pelleted by centrifugation (4000rpm, 10min, 4°C) in a 50ml Sorval tube. The pellet was resuspended in 5ml of Solution I, followed by 10ml of Solution II and the tube was gently rolled until the solution became viscous and clear. After 5 minutes at room temperature, 5ml of Solution III was added and the solution thoroughly mixed to break up the precipitate. The precipitate was pelleted by centrifugation (10000rpm, 20min, 4°C) and the supernatant strained through sterile muslin into a fresh tube.

The DNA was precipitated from the supernatant by adding an equal volume of cold isopropanol and placing the tube on ice for 10 minutes. Once pelleted by centrifugation (2500rpm, 10min, 4°C) and the supernatant removed by suction, the DNA was dissolved in 1ml of TE and then transferred to a 10ml centrifuge tube where an equal volume of 5M LiCl was added. After 10 minutes on ice, the precipitate was removed by centrifugation and the supernatant transferred into a clean tube. The DNA was precipitated with cold isopropanol, pelleted and dissolved in 0.4ml of TE. After transferring the supernatant to an eppendorf tube, 2µl of RNaseA (10mg/ml) was added and the tube incubated at 37°C for 15 minutes. The DNA was precipitated by adding 500µl of 2.5M NaCl/20% PEG and placing the tube at -20°C for 10 minutes. Once pelleted and dried, the DNA was dissolved in 250µl of TE.

Contaminants were extracted from the DNA using an equal volume of phenol:isoamyl alchohol (24:1) and chloroform, followed by two chloroform extractions to remove any remaining phenol. The DNA was precipitated using ethanol (Methods 2.4.4) and pelleted by centrifugation. If no precipitate could be seen the sample was left at -20°C overnight before the DNA was centrifuged. Once the supernatant was

discarded, the pellet was washed with 1ml of 70% ethanol, vacuum dried and dissolved in 200µl of sterile distilled water.

2.4.4 ETHANOL PRECIPITATION OF DNA.

1/10 volume of 3M sodium acetate (pH 4.8) was added to the DNA sample followed by 2 volumes of 100% ethanol. After 10 minutes at -80°C the DNA was pelleted by centrifugation, the supernatant removed and the pellet was redissolved in an appropriate volume of sterile glass distilled water or TE.

2.4.5 RESTRICTION DIGESTION OF DNA.

Restriction digestions were carried out using the manufacturers' recommended conditions and buffers. Typically, reactions were carried out in 10-20µl volumes and incubated for 4 hours or overnight. For double digestions, the most suitable buffer for the two enzymes was used. In some cases the enzyme with the lowest salt requirement was incubated first, then the second enzyme and more salt were added and the incubation continued at the appropriate temperature.

2.4.6 GEL ELECTROPHORESIS.

Agarose dissolved in 1x TAE buffer was used for gel electrophoresis. The agarose content varied between 0.5-1.5% depending on the size of the fragment of interest. Ethidium bromide (0.5µg/ml) was used to stain the gels for 20 minutes, followed by destaining in water for 5-10 minutes if required. DNA bands were visualised using a Sigma T2210 UV Transilluminator (302nm).

2.4.7 RNaseA TREATMENT OF DNA FOR GEL ELECTROPHORESIS.

Loading buffer containing RNaseA (10µg/ml) was added to the DNA and the sample was incubated at 37°C for 5 minutes before loading onto the agarose gel. Treatment after gel electrophoresis could be achieved by soaking the gel in RNaseA solution (10µg/ml in 1xTAE) at 37°C for 15 minutes.

2.4.8 ELUTION OF DNA FRAGMENTS FROM AGAROSE GELS: MODIFIED SPIN-DOWN METHOD (He et al., *1992).*

A 1ml Gilson blue tip was cut, the end plugged with glass wool and then placed inside an eppendorf tube. DNA fragments were separated by electrophoresis and the gel stained briefly in fresh ethidium bromide. The DNA fragment was visualised using a UV transilluminator, excised from the gel using a razor blade and placed onto a piece of Whatman 3MM paper to reduce the volume of eluted DNA solution. Once placed inside the blue tip, the eppendorf tube was centrifuged (6000rpm, 2min) and the eluted DNA collected. The DNA fragment was then used directly in further manipulations or ethanol precipitated to concentrate the sample before further use.

2.4.9 DEPHOSPHORYLATION OF LINEARISED PLASMID DNA USING CALF INTESTINAL ALKALINE PHOSPHATASE (CIP) (Sambrook et al., *1989).*

Following restriction digestion of DNA (1μg), the restriction enzymes were inactivated by heating and the DNA was precipitatedwith ethanol. The DNA was dissolved in 90μl 10mM Tris (pH 8.3) and 10μl of 10x CIP dephosphorylation buffer added along with 1μl of CIP. After a one hour incubation at 37°C, EDTA (5mM) was added and the CIP was heat inactivated. The DNA was extracted once with phenol:chloroform (1:1) and once with chloroform followed by ethanol precipitation. Centrifugation was used to pellet the DNA, which was then resuspended in 10μl of sterile glass distilled water. The DNA concentration was determined by electrophoresis.

2.4.10 PARTIAL DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES (Ausubel et al., *1989).*

Partial digestion was used to clone segments of DNA where the desired restriction site were represented many times within the fragment. A 100μl reaction mixture containing DNA (3μg) in 1x restriction buffer was prepared and divided into five microfuge tubes so that tube one contained 45μl, tube two 20μl, tube three 15μl and tubes four and five 10μl each. The restriction enzyme (5 units) was added to tube one, and a 20μl and 5μl aliquot was transferred to tubes two and four respectively. A 15μl and 5μl aliquot from tube two was then transferred to tubes three and five respectively. All the tubes were

incubated at 37°C for 15 minutes and the reaction stopped heat inactivating the enzyme. The extent of digestion was checked by gel electrophoresis using a 10µl sample from each reaction. The remaining digest was then ligated to the appropriately digested vector using T4 DNA Ligase in 1x Ligation Buffer.

2.4.11 LIGATION OF DNA.

Ligation was performed in a total volume of 20µl with 4µl of BRL 5x Ligation Buffer. As a common practice, a 3:1 insert to vector ratio was used. Before the DNA was added to the ligation reaction, the restriction enzyme was heat inactivated. One unit of BRL T4 Ligase was used for each ligation and the reaction mix incubated at 16°C or room temperature overnight. The ligation efficiency was checked by gel electrophoresis.

2.4.12 ELECTROPORATION.

Preparation of Competent Cells.

An exponential culture of MC4100 was grown in 200ml of LB broth, using 2ml of an overnight LB broth culture as the inoculum. The culture was shaken at 37°C until the cells reached an OD_{600} of 0.5-0.8. After chilling the culture on ice for 30 minutes, the cells were collected by centrifugation (4000rpm, 15min, 4°C), suspended in 200ml of high quality distilled water and placed on ice 30 minutes. This step was repeated and the pellet suspended in 100ml. Again the cells were collected by centrifugation and the pellet resuspended in 4ml of ice cold 10% glycerol. After a 30 minute incubation on ice and a final centrifugation step, the pellet was suspended in 400µl of ice cold 10% glycerol and 40µl aliquots were either used immediately or stored at -80°C until required. When required, frozen cells were first thawed on ice.

Electroporation.

Sterile electroporation cuvettes (0.2cm gap) and the chamber slide were chilled on ice and the Biorad Gene Pulser set to 25MF and 250kV and the Pulse Controller set to 200ohms. Approximately 100ng of DNA was added to the thawed competent cells and the mixture left on ice for one minute. The mixture was transferred to the chilled cuvette which was then placed into the chamber slide. Once the chamber slide was placed into the electroporation chamber the sample was pulsed once. A 1ml aliquot of SOC media was added immediately to the sample and the supension transferred to an eppendorf tube. The cells were elaborated in the eppendorf tube at 37°C for 1-2 hours and a 200µl aliquot was then plated on an LB+antibiotics plate. After harvesting the cells by centrifugation, the cells were resuspended in 200µl of LB broth and equally spread onto two LB+antibiotic plates. The plates were then incubated overnight.

2.4.13 ELECTROPORATION OF LIGATED DNA INTO BACTERIA

(Zabarovsky and Winberg, 1990).

Ligation was performed in a 20µl volume as previously described. Potassium acetate (pH 8.0) was added to a final concentration of 0.25M along with 2.5 volumes of 100% ethanol. Precipitates were allowed to form at -20°C for two hours and the DNA pelleted by centrifugation. After two rinses with 70% ethanol, the pellet was redissolved in 5µl sterile distilled water. A 1-2µl aliquot was then used in the electroporation step as previously described.

2.4.14 PREPARATION OF COMPETENT CELLS.

Exponentially grown cells were harvested by centrifugation and the supernatant removed. The cells were suspended in cold Solution I (1/2 the original volume) and placed on ice for 20 minutes. This step was repeated and the pellet suspended in cold Solution II (1/4 the original volume) and placed on ice. Following centrifugation and removal of the supernatant, the pellet was gently suspended in cold Solution II (1/15 the original volume). After 30 minutes on ice, 200µl aliquots of cells were used immediately or stored at -80°C, after the addition of 7µl of 99.5% dimethyl sulfoxide.

2.4.15 TRANSFORMATION.

Approximately 100-200ng of DNA was added to thawed competent cells and gently mixed. After 30 minutes on ice, the mixture was transferred to a 42°C heating block for 2 minutes and then placed on ice immediately. The cells were elaborated and plated on LB+antibiotic plates as previously described (Methods 2.4.12).

2.4.16 NESTED DELETIONS OF CLONED DNA.

Nested deletions of cloned DNA were generated using the Deletion Factory™ System (Life Technologies, 8378SA). The DNA fragment of interest was cloned into the deletion vector pDELTA 1 using the techniques previously described and electroporated into *E. coli* DH10B. Transformants were selected on LB+X-gal+Tet+Kan plates, with white colonies denoting clones. Single colonies were then cultured in 2ml LB+Tet+Kan, the plasmid DNA extracted and the correct insert verified by restriction digestion and agarose gel electrophoresis. The correct plasmid was transformed into *E.coli* DF1 which could provide the transposase gene in *trans*. After transformants were selected on LB+Tet+Kan+Cm plates, five colonies were transferred to a flask containing 2ml LB+Cm+Tet and five to a flask containing 2ml LB+Cm+Kan. After an overnight incubation, plasmid DNA was extracted from each culture and an aliquot checked by gel electrophoresis. The DNA was diluted 1\10-1\20 and 1µl electroporated into DH10B with selection on LB+Tet+Sucrose (Cm+Tet) and LB+Kan+Strep (Cm+Kan). Resulting transformants were purified and the plasmid DNA examined by restriction digestion and agarose gel electrophoresis to determine the size of the deletion. Deleted plasmids with the desired sized inserts were then chosen for further analysis by DNA sequencing.

2.5 MUTAGENESIS.

2.5.1 MUTAGENESIS USING ALTERED TARGET SPECIFICITY MINI-TN10 DERIVATIVES (Kleckner et al., *1991).*

Preparation of High Titre Phage.

Phage stock was diluted to 10^{-6} and 100µl added to 3ml of molten H-Top agar, seeded with 100µl of PB2480 overnight culture (grown in TBMM containing 0.2% maltose). The H-Top agar was poured over a Tryptone plate, left to set and the plate incubated overnight.

Using a Pasteur pipette, a single plaque was removed from the Tryptone plate and added to a 100ml flask containing 10ml LB broth+0.01mM $MgSO_4$ and 100µl of a PB2480 overnight culture. The flask was shaken vigorously at 37°C for 4-5 hours until lysis had occured, 10 drops of chloroform were added and the flask shaken and left to settle. The cellular debris were removed by centrifugation (6000rpm, 10min, 4°C) and the supernatant poured into McCartney bottles to be stored at 4°C after adding 5 drops of chloroform.

Determining Phage Titre.

A 2µl aliquot of phage stock was diluted in a series of 100-fold dilutions to 10^{-8}. An LB plate was overlayed with PB2480 and 10µl from each dilution spotted onto the lawn. After drying, the plate was incubated overnight and the number of plaques in each dilution counted. Phage titre = number of plaques x dilution x volume correction.

Transduction of mini-Tn*10*Kan.

An overnight culture of the bacteria to be mutated when grown in 5ml TBMM with the appropriate antibiotics was estimated to contain approximately $1x10^9$ cells/ml. Once harvested by centrifugation, the cells were suspended in TBMM (1/10 the original volume) to give a final concentration of $1x10^{10}$ cells/ml. Aliquots of 100µl were placed into five eppendorf tubes and phage stock added to a m.o.i of 0.3. The mixture was incubated at 25°C for 15 minutes and then at 37°C for 15 minutes. Unadsorbed phage were removed by washing the culture in 5ml of LB broth containing 50mM sodium

citrate and the cells collected by centrifugation. The cells were suspended in 3ml of LB broth containing 50mM sodium citrate and incubated at 37°C. After 90 minutes, 200µl of cells were plated out on a LB+Kan+Napp plate and the remaining culture pelleted by centrifugation. Once the supernatant was removed, the pellet was suspended in 1ml of LB broth and 100µl aliquots spread onto each of three LB+Kan+Napp plates. After an overnight incubation, the colonies were resuspended in 3ml of LB broth and the suspension used as the starting material for plasmid DNA extraction. Mini-Tn*10* insertions into plasmids were then selected by electroporating the plasmid DNA into *E. coli* MC4100.

2.5.2 GENERATION OF TRANSLATIONAL FUSIONS (Kleckner et al., *1991).*

Translational fusions between *lacZ'* and the target gene were created using the 4.9kb mini-Tn*10lacZ'* Kan^Rderivative 112 carried on λ NK1205 (Kleckner *et al.*, 1991). The strain carrying the plasmid of interest was first transformed with pNK2882 (*tnpA* Tet^R) using electoporation to provide the transposase function in *trans*. The preparation of a high titre lysate and the transduction of λ NK1205 was performed as previously described in Methods 2.5.1. A 1µl aliquot of plasmid DNA from each pool was electroporated into MC4100 and plated on LB+Amp+Kan+X-gal+IPTG plates. The resulting transformants were then screened for microcin production, β-galactosidase activity and sensitivity to tetracycline.

2.5.3 DETERMINING GENE EXPRESSION BY β*-GALACTOSIDASE ACTIVITY (Miller 1972; Sambrook* et al., *1989).*

An overnight culture was grown in 1x A medium containing 0.4% glucose, 1µg/ml vitamin B_1, 1mM $MgSO_4$ and the appropriate antibiotic. The next day the culture was diluted 1:50 in the same medium, grown until it reached an A_{600} of 0.4 and then placed on ice. A 100µl aliquot of culture was added to 900µl of Z buffer and two drops of chloroform and one drop of 0.1% SDS were added. After 10 seconds vortexing, the lysed culture was equilibrated to 30°C for 10 minutes and then 200µl ONPG in A medium (4mg/ml) was added. In cases where low levels of β-galactosidase activity were expected, 500µl of culture and 500µl of Z buffer was used. The reaction sample was left until a yellow colour developed, the reaction was then stopped by adding 500µl

of 1M Na_2CO_3 and the time in minutes recorded. The A_{600} of the culture and the A_{420} and A_{550} of the reaction sample were determined using a LKB Ultraspec II and the units of β-galactosidase activity calculated. The A_{550} reading was ignored if the reaction sample was centrifuged prior to reading.

2.6. DNA SEQUENCING OF DOUBLE STRANDED DNA TEMPLATES.

DNA sequencing of double stranded templates was performed using the dideoxy chain termination method (Sanger *et al.*, 1977). Sequence reactions were carried out with [^{35}S]dATPαS (Amersham, SJ1304) using the T7 Sequencing™ Kit (Pharmacia Biotech, 27-1682-01).

2.6.1 TEMPLATE PREPARATION (Modification to T7 Sequencing manual).

A single bacterial colony containing the nested deletion was used to inoculate 10ml 2xYT media with the appropriate antibiotic and incubated overnight. The cells were harvested by centrifugation and resuspended in 1ml Solution I. After 10 minutes at room temperature, 1ml of Solution II was added followed by a further 10 minute incubation. Solution III (1ml) was then added and the tubes placed on ice for 10 minutes. The precipitate was pelleted by centrifugation and the supernatant drained through nappy liner. An equal volume of cold isopropanol was added to the sample and the mixture left at room temperature for five minutes. The DNA was pelleted by centrifugation (12000rpm, 4°C, 10min.), the supernatant removed by aspiration and the pellet resuspended in 200µl distilled water. RNA was removed by adding 20µl RNase A (20mg\ml) and incubating at 37°C for 20 minutes. Two phenol/ chloroform extractions followed by a chloroform extraction were then performed and the DNA ethanol precipitated. The DNA was dissolved in 50µl distilled water and a 1µl aliquot was digested with the appropriate restriction enzyme and analysed by agarose gel electrophoresis. If required 0.5µl RNase A was then added.

2.6.2 PRIMER ANNEALING.

Template DNA (2μg) was added to 8μl 2M NaOH and the volume adjusted to 40μl with sterile distilled water. After vortexing and brief centrifugation, the DNA was incubated at room temperature for 10 minutes, 7μl of 3M sodium acetate, 4μl distilled water and 120μl 100% ethanol were added and the solution left at -20°C overnight. The DNA was collected by centrifugation, washed in 1ml 70% ethanol and spun for a further 10 minutes. Once the supernatant was removed by aspiration and the pellet dried under vacuum, the DNA was dissolved in 9μl distilled water, 3μl primer (10mg/ml) and 2μl annealing buffer. After vortexing and pulse centrifugation the template was incubated at 65°C for five minutes, 37°C for ten minutes and room temperature for at least 5 minutes. If the template was not going to be used immediately it was stored at -20°C.

2.6.3 LABELLING AND TERMINATION REACTIONS.

Prior to labelling the templates, four microfuge tubes were labelled A, C, G, T and 2.5μl of the appropriate termination mix (short reaction) added. T7 DNA polymerase was diluted to 1.6units/μl and 2μl added to the annealed template plus 3μl of labelling mix-dATP and 0.5-1μl [α-^{35}S]dATPαS. After 5 minutes at room temperature, 4.5μl was removed and added to each of the four termination mix tubes followed by a 5 minute incubation at 37°C. A 5μl aliquot of stop buffer was added to each tube and the tubes heated to 85°C for 20 minutes prior to electrophoresis.

2.6.4 ELECTROPHORESIS AND AUTORADIOGRAPHY.

The LKB 2010 Macrophor sequencing system was used to make and run the sequencing gels. Sequencing gels (6% acrylamide, 0.2mm) were poured using the LKB 2010-001 macromould gel casting unit. Pre-electrophoresis of the gel was performed for 30 minutes at 2000 volts. After heating the sequencing reactions, 0.5-1μl was loaded into the appropriate well and the gel run at 1500-2000 volts for 2.5-3 hours. The remaining sample was stored at -20°C and if required for another gel, was heated to 85°C for five minutes prior to electrophoresis.

Once electrophoresis was completed, the notched plate with the gel attached was carefully removed from the thermostatic plate and placed in 10% acetic acid for 20

minutes and dried at 65°C for 60 minutes. After leaving the plate to cool, Hyperpaper (Amersham RPN1804) was cut to size and placed emulsion side down on the gel. A second glass plate was placed on top, clamped and the sandwich was put in a light-proof box for 16-20 hours to expose the Hyperpaper. The Hyperpaper was developed in Agfa G150 Developer for 3 minutes, washed in water for 2 minutes and the image fixed in Agfa Fixer for 5 minutes.

2.7. PROTEIN ANALYSIS USING SDS-PAGE.

2.7.1 SDS-PAGE (Modification of Laemmli, 1970).

Denaturing protein gels were made according to Hoefer Scientific Instruments, Sturdier Slab Gel Electrophoresis Unit instructions. All buffers and gel formulae are given in Appendix 1.

The resolving gel was prepared as described and poured between two, 150mm glass plates set 1.5mm apart. To aid polymerisation, water-saturated butanol was placed on top of the resolving gel. After polymerisation and removal of the butanol, the interface was rinsed with distilled water, the stacking gel was poured and the well former inserted. The gel was left to set for one hour, attached to a vertical electrophoresis box and the reservoirs filled with Tank Buffer. Between 50-100µl of sample was loaded into each well and electrophoresis performed at 20mA through the stacking gel and then 30-40mA until the Bromphenol Blue dye front reached the bottom of the gel.

The gel was stained in Coomassie Brilliant Blue (R-250) for 2-4 hours and destained in Solution 1 overnight prior to further treatment if required. Destain Solution 2 could also be used for the overnight soak.

2.7.2 SDS-PAGE OF RADIOACTIVELY LABELLED PROTEINS.

The gel was prepared as previously described with the plates set 0.75mm apart. After electrophoresis, the gel was stained in Commassie Blue for 2 hours, destained for 2 hours and then soaked overnight in predrying solution (3% glycerol, 40% methanol, 10% acetic acid). After a 30 minute wash in distilled water, the gel was placed in Amplify™ (Amersham) for 30 minutes and then placed in distilled water. The gel was

vacuum dried onto Whatman 3MM at 60°C for 2-3 hours using a Model 443 Slab drier (Bio Rad) and then placed in an autoradiography cassette containing an intensifying screen.

Autoradiography.

Amersham Hyperfilm-MP was cut to size and placed in the cassette with the dried gel and exposed at -80°C for 2-7 days. The film was developed in Agfa G150 Developer for five minutes, rinsed in water for two minutes and then fixed in Agfa Fixer for eight minutes. After a final wash under running water the film was left to dry.

2.7.3 SDS-PAGE MAXIMISED FOR SMALL PROTEINS (Thomas and Kornberg, 1978).

Visualisation of small peptides was maximised using the method of Thomas and Kornberg 1978. The procedure is the same as that described in Methods 2.7.2 with the following modifications: an 18% polyacrylamide separating gel and 3% stacking gel were used; the concentration of Tris buffer in the separating gel was raised to 0.75*M*; the ratio *N,N'*-methylene bisacrylamide:acrylamide was lowered to 0.15:30; the electrode buffer was changed to 50mM Tris, 0.38M glycine and 0.1% (w/v) SDS.

The resolving gel was prepared as described in Appendix 1 and the gel poured to a depth of 10-15cm and a width of 0.75mm. The gel was covered with water-saturated butanol and left to set, after which the butanol was replaced with 0.1% SDS and the gel left for approximately one hour. The stacking gel was prepared as described (Appendix 1), the well forming comb inserted to a depth of half the stacking gel and the gel left to set for 30 minutes.

The samples were applied to the gel and electrophoresis was performed at a constant current of 30mA until the dye ran off the end of the gel after approximately five hours. Staining, drying and autoradiography were performed as previously described (Methods 2.7.2). The Storm™ 840 PhosphorImager (Molecular Dynamics) was used as an alternative to normal autoradiography. A dried gel was wrapped in clingwrap, placed in a phosphorscreen cassette and exposed for 3-7 days. The phosphorimager was then used to read the image from the phosphorscreen.

2.7.4 SILVER STAINING OF SDS-PAGE GELS.

The SDS-PAGE gel was washed with agitation in Wash Solution 1 for 30 minutes. After a 15 minute wash in distilled water, the previous step was repeated using Wash Solution 2. Distilled water was used to rinse the gel for 10 min, fresh distilled water added and the rinse repeated. The gel was left agitating in distilled water overnight.

Fresh stain was made and the gel stained for 10 minutes while being agitated. The gel was washed in distilled water for 5 minutes and the wash repeated 3 times. Fresh developer was prepared and the gel soaked with agitation until the protein bands were visible, then the gel was quickly placed in fixer solution. Leaving the gel in fixer solution overnight enhanced the contrast of the protein bands.

2.8. EXPRESSION OF PLASMID ENCODED PROTEINS.

2.8.1 MINICELL ANALYSIS OF PLASMID ENCODED PROTEINS (Clark-Curtiss and Curtiss III, 1983; Rodriguez and Tait, 1983).

Preparation of *E. coli* mini-cells.

The minicell producing strain *E. coli* P678-54T was made competent for electroporation as previously described and transformed with the desired plasmids. DNA was prepared from the transformants by alkaline extraction and the presence of the correct plasmid was confirmed by restriction digestion and electrophoresis.

Plasmid-containing minicells were prepared by differential rate centrifugation through sucrose gradients. A 20% sucrose solution was made in M63 salts and 35ml aliquots were frozen at -80°C for 1-2 hours in 50ml centrifuge tubes. Prior to use, the tubes were thawed overnight at 4°C to form the gradient.

A 300ml culture of the plasmid containing minicell strain was grown overnight in LB broth containing the appropriate antibiotics until it reached an OD_{600} = 0.8 - 1.2. The vegetative cells were collected by centrifugation (2000rpm, 10min, 4°C) and the supernatant containing the minicells was poured off into a new tube. A small amount of the remaining supernatant was gently pipetted over the vegetative pellet to remove any minicells and the liquid added to the initial supernatant fraction. The minicells were harvested by centrifugation (10,000rpm, 10min, 4°C), the supernatant was poured off

and the pellet resuspended in 2ml BSG buffer. Once resuspended the cells were gently pipetted onto the surface of the sucrose gradient and the tubes spun for 10 minutes (5000rpm, 10°C) using a swing out rotor. The top half of the minicell band , 5-10ml, was removed and added to an equal volume of BSG buffer. After re-centrifugation (10,000rpm, 10min 4°C), the cells were resuspended in 2ml BSG buffer and loaded onto a second sucrose gradient and centrifuged as before. After harvesting the top half of the minicell band and resedimentation as previously described, the pellet was resuspended in 10ml BSG buffer. The OD_{600} of the minicells were measured, the cells pelleted by centrifugation and the pellet resuspended in M63/30% glycerol to give an $OD_{600} = 2.0$. The cells were stored in 100µl aliquots at -80°C until required. To test for vegetative cell contamination, a 10µl aliquot of cells was spread on a LB plate containing the appropriate antibiotics and incubated overnight. Fewer than 1000 colonies per 10µl was considered an acceptable level of contamination.

Labelling and electrophoresis of plasmid-encoded proteins.

Each 100µl aliquot of frozen minicells were thawed on ice for 20 minutes and 900µl of M63 labelling buffer added. The cells were incubated in a 25ml flask for 30 minutes and 2µl of ^{35}S-methionine/cysteine (20mCi/ml, PRO-MIX, Amersham SJQ0079) was added. After a further 40 minutes incubation, the cells were pelleted in a microfuge tube by centrifugation (12,000rpm, 2min, 4°C) and resuspended in 60µl Storage Buffer. The labelled minicells were stored at -20°C until required. Labelled minicells were thawed on ice and 60µl of 2x Treatment Buffer added. The samples were placed in a boiling water bath for 3 minutes, and a 60µl aliquot was analysed by SDS-PAGE as previously described (Methods 2.7.2).

2.8.2 MAXICELL ANALYSIS OF PLASMID ENCODED PROTEINS (modified from Pritchard and Holland, 1985).

UV-irradiation dose test.

Individual 10ml cultures of MC4100*recA*-56 and MC4100(pBR322) were made in M63 medium+antibiotics from a 1/100th dilution of a M63 overnight culture and grown at 37^{o}C to an absorbance (450nm) of 0.50. A UVC-515 Ultraviolet Multilinker (UltraLum, 254nm) was used to irradiate 1ml aliquots of culture at 100, 136, 150, 200, 250, 300, 400 and 500μJcm^{-2}. The cells were diluted by factors of 10 down to 10^{-8} and 10μl spots of each dilution were dried onto LB plates and incubated overnight. The smallest UV dose which prevented growth of both MC4100 and MC4100(pBR322) was used to generate maxi-cells.

Generation of maxi-cells.

The plasmids to be analysed were first electroporated into MC4100*recA*-56 and the presence of the plasmids confirmed by DNA extraction and restriction analysis. A 3ml culture of the plasmid-containing MC4100 strain was grown in M63 medium+antibiotics overnight, and a 1/100th dilution used to seed 10ml of M63+antibiotics+AAA-met/cys. The culture was grown to an absorbance (450nm) of 0.5 and 3.5ml placed in a sterile petrie dish. The cells were irradiated with UV (200μJcm^{-2}) and placed in a foil covered sterile flask. A 10μl aliquot was plated onto a LB plate, wrapped in foil and incubated overnight to indicate the effectiveness of irradiating the cells. The culture was incubated with agitation for one hour, D-cycloserine (100μg/ml) was added and the culture incubated overnight. A 100μl aliquot of cells was then plated onto a LB plate to show the number of viable cells.

Labelling and electrophoresis of plasmid encoded proteins.

A 0.5ml aliquot of cells was harvested by centrifugation (6000rpm, 4^{o}C, 10min) washed twice with 0.5ml 1xM63 salts and resuspended in 200μl M63 medium+0.4% glucose+AAA-met/cys. Following a one hour incubation, ^{35}S-methionine/cysteine (PRO-MIX, 20mCi/ml) was added to a concentration of 20-40μCi/ml. The cells were incubated for 15-30 minutes, after which 10μl of both methionine (2mg/ml) and cysteine (2mg/ml) were added and the cells incubated for five minutes. The cells were

harvested, washed twice with 1xM63 salts and resuspended in 30µl TE. An equal volume of 2x Treatment Buffer was added and the tube placed in a boiling water bath for five minutes prior to electrophoresis. Samples (20-30µl) were analysed by SDS-PAGE maximised for small proteins (Methods 2.7.3), or frozen at -20°C until required.

2.9 MICROCIN EXTRACTION TECHNIQUES.

The microcin titre in all supernatants and extracts was estimated using the critical dilution method (Methods, 2.10.1). At the end of each extraction procedure, protein profiles were determined using SDS-PAGE (Methods 2.7.1).

2.9.1 EXPRESSION OF MICROCIN 24 USING MITOMYCIN C (Pugsley and Oudega 1987).

An exponential culture of *E. coli* MC4100(pGOB18) in LB broth was grown with aeration until it reached an OD_{600} of 0.2. Mitomycin C was added to a concentration of 0.5µg/ml and the culture shaken for 2-5 hours. The supernatant was collected after two centrifugation steps (10000rpm, 10min, 4°C). EDTA and sodium azide were added to a final concentration of 5mM and 0.02% (w/v) respectively and the supernatant stored at 4°C. The supernatant was either used for various extraction procedures, concentrated in a vacuum centrifuge (Jouan, RC10.10), or acetone precipitated.

Protein profiles were determined by SDS-PAGE after 2x Treatment Buffer was added to the samples in a ratio of 1:1. Once placed in a boiling water bath for 90 seconds, the samples were loaded into the gel.

2.9.2 EXPRESSION OF MICROCIN 24 USING M63 MEDIUM.

An overnight culture of MC4100(pGOB18) was grown in M63 medium+Amp and diluted 1/100 into 2x 500ml of the same medium. The culture was grown to stationary phase (6-8 hours), chloroform added and the cells removed by centrifugation (11,000rpm, 10 min, 4°C). Mcc24 was extracted by one of the methods described (Methods 2.9.3-2.9.7) and the microcin titre estimated.

2.9.3 ACETONE PRECIPITATION OF MICROCIN 24 EXTRACTS (Pugsley and Oudega, 1987).

Two volumes of ice cold acetone were added one volume at a time, to prepared microcin extracts or spent supernatants, and the solution frozen at -80°C for 15 minutes. The protein was collected by centrifugation (11,000rpm, 10 min, 4°C), the acetone removed by aspiration and the pellet dried in a vacuum desicator. The protein pellet was redissolved in an appropriate buffer and assayed for microcin activity and the protein profile determined.

2.9.4 AMMONIUM SULPHATE PRECIPITATION (Englard and Seifter, 1990).

Production of Mcc24 from a 50ml culture was induced using mitomycin C and the supernatant used for precipitation. Ammonium sulphate was added to the supernatant to a saturation of 25%, stired at 0°C until dissolved and left to sit for 15 min. The precipitate was collected by centrifugation (12,000rpm, 0°C, 10 min) and the supernatant decanted to form the starting material for the next fraction. Ammonium sulphate was added to final saturations of 40%, 50%, 60% 80% and 100% and the procedure repeated each time. The precipitate collected after each fractionation was redissolved in 3ml of 50mM KH_2PO_4 buffer, acetone precipitated and redissolved in 200µl of phosphate buffer. Microcin titers and protein profiles were determined as previously described.

2.9.5 BULK PREPARATION OF MICROCIN 24 BY FILTRATION THROUGH NITROCELLULOSE (Pugsley and Oudega, 1987).

The production of Mcc24 was induced using M63 broth cultures (Methods 2.9.2). After the removal of cellular material by centrifugation, each 400ml supernatant was filtered under vacuum through two nitrocellulose filters (0.22µm, Millipore) using a Buchner funnel. The filters were washed in a McCartney bottle with 8 ml of Wash Buffer and then transferred to another bottle containing 8ml of Elution Buffer. After vortexing, the filters were removed and the protein precipitated using acetone. The protein pellet was dissolved in 1ml of Wash buffer after being air dried. Activity and protein profiles of the extract were then determined.

2.9.6 GEL FILTRATION OF CRUDE MICROCIN 24 EXTRACT (Pharmacia Gel Filtration Handbook).

Preparation of Column.

Sephadex G-100 (15g) was soaked in excess buffer for 24 hours. Gas was removed from the Sephadex G-100 under vacuum and the matrix gently poured into a SR25 column (Pharmacia) to a height of 30cm. After equilibriating the gel with three column volumes of buffer, the gel was calibrated using 500µl of Blue Dextran dye. The miniscus of the dye was allowed to enter the column, the dye washed through the column with excess buffer and collected using a measuring cylinder. The void volumn, (V_0), was determined as the volume of buffer passing through the column when the Blue Dextran was first washed out. Blue Dextran was removed from the column by 2 column volumes of buffer.

Fractionation.

Mcc24 extracts were prepared from LB broth cultures (Methods 2.9.1) and concentrated to 1-2ml by acteone precipitation. Sucrose was added to the sample giving a final concentration of 20%. The volume of Mcc24 extract loaded onto the column did not exceed 1-5% of the bed volume. The buffer was run to just above the top of the gel, then 2ml of crude extract was added to the column using a Pasteur pipette and being careful to not disturb the top of the column. Buffer was added to the column, the column attached to a UA-5™ Absorbance Detector (280nm, ISCO) and Foxy™ Fraction Collector (ISCO) and the buffer passed through the column overnight at a flow rate of 600µl/min using a TRIS™ Peristaltic Pump (ISCO). Fractions containing 4.125ml (75 drops) were collect after one V_0 volume had passed through the column and the fractions concentrated to 1ml using a vacuum centrifuge (Jouan, RC10.10).

Column Storage.

After the samples were collected, the column was washed with one volume of 0.2M NaOH and then two volumes of buffer. The column was stored in buffer containing 0.02% sodium azide. Before further use, the column was washed with 2 volumes of buffer.

Sample Assay.

A 3µl aliquot from each fraction was spotted onto a lawn of MC4100 and the plate incubated for 4-8 hours. Clear zones in the lawn denoted Mcc24 activity in those samples. Protein profiles of active fractions were examined using SDS-PAGE.

2.9.7 ION EXCHANGE CHROMATOGRAPHY (HiTrap™ Instruction Manual, Pharmacia).

Microcin 24 extracts were prepared as described (Methods 2.9.1) and the supernatant was placed into sterile dialysis tubing and dialysed against the desired buffer for 12-24 hours at 4°C with at least two changes of buffer. The dialysed supernatant was transfered to a McCarney bottle and assayed for activity.

The anion exchange column (Q) or the cation exchange column (SP) were attached to a peristaltic pump and prepared by washing with five column volumes of Start buffer, five column volumes of Elution buffer and a further five column volumes of Start buffer at a flow rate of 1ml/min. The dialysed sample was applied to the column using the peristaltic pump and the eluate collected. The column was then washed with five column volumes of Start buffer, the eluate collected and the protein extracted with three column volumes of Elution buffer. The eluate at each step was precipitated with acetone, the precipitate dissolved in 100µl of Start buffer and microcin activity tested using the critical dilution method (Methods, 2.10.1). The protein profile of each fraction was determined by SDS-PAGE.

The columns were cleaned with five column volumes of Elution buffer followed by Start buffer and stored in 20% ethanol/0.2M sodium acetate (SP) or 20% ethanol (Q).

2.10 MICROCIN 24 ACTIVITY TECHNIQUES.

2.10.1 ESTIMATION OF MICROCIN TITRE - CRITICAL DILUTION METHOD (Mayr-Harting et al., *1972).*

The microcin titre of supernatants or concentrated extracts was estimated by making doubling dilutions in 0.1M phosphate buffer (pH 7.4) down to 1:1024. A MA plate was overlayed as previously described (Methods 2.3.3) and a 5µl aliquot from each dilution

was spotted onto the lawn and the plate incubated overnight. The microcin titre was the reciprocal of the last dilution giving a clear zone of inhibition. The units for microcin titre are Arbitrary Units (A.U.) per millilitre.

2.10.2 MICROCIN ACTIVITY AFTER SDS-PAGE (Fath et al., *1994).*

Mcc24 extracts were prepared and analysed by SDS-PAGE after the samples were heated to 42°C for 15 minutes before loading onto the gel. The gel was divided into two equal halves and samples run in both halves. One half of the gel was silver stained and the other half was soaked in 1x M63 medium for 1-2 hours. The gel which was soaked was placed on a MA plate and overlayed with MC4100 in H-Top agar. Microcin activity was determined by looking for inhibition of MC4100 growth.

2.10.3 EFFECT OF CHLOROFORM ON MICROCIN 24 ACTIVITY.

An overnight culture of MC4100(pGOB18) was grown in LB broth and 100µl placed into each of four microfuge tubes. Into three of the tubes was added either 10µl chloroform, 5µl mitomycin C (0.1µg/ml) or 10µl chloroform + 5µl mitomycin C and the fourth tube kept as a control. The cells were incubated for one hour and then harvested by centrifugation (13,000rpm, 4°C, 10 min). A 20µl aliquot of the supernatant was placed onto a lawn of MC4100, dried and microcin activity scored after 4-6 hours incubation at 37°C.

2.10.4 PROTEINASES OR RNase TREATMENT OF MICROCIN 24.

Circular paper filters (1cm diameter) were soaked in Proteinase K (1mg/ml) or RNase A (10mg/ml) solutions. The filters were placed on a M63 plate and overlayed with 4ml of H-Top agar seeded with 100µl of MC4100. Colonies of MC4100(pGOB18) were toothpicked onto, or at various distances from the filters, and the plates incubated overnight. An absence of a halo around MC4100(pGOB18) on the MC4100 lawn was seen as inhibition of Mcc24 activity by Proteinase K or RNase A.

2.10.5 TIME SERIES FOR MICROCIN 24 ACTIVITY.

An overnight culture of MC4100 was grown in M63 medium, diluted 1/100 and grown until the culture reached an OD_{600} of 0.2. The Mcc24 extract was prepared from 3x

400ml M63 medium cultures using nitrocellulose extraction (Methods 2.9.5) and the protein dissolved in 750µl of 50mM Phosphate Buffer (pH 7.4). A 50µl aliquot of cells was placed in each of six eppendorf tubes and 50µl of microcin extract was added. The tubes were incubated for 0, 15, 30, 60, 120 and 360 minutes with one tube at each time point removed. The cells were harvested by centrifugation, resuspended in 50µl LB broth and diluted by factors of ten to 10^{-8}. After spotting 10µl from each dilution onto an LB plate, the cells were grown overnight, colonies counted and cell concentrations determined. The activity of the microcin extract was also determined using the critical dilution method.

2.10.7 EFFECT OF MICROCIN 24 ON AMINO ACID UPTAKE (Pugsley and Oudega, 1987).

An overnight culture of MC4100 was grown in M63 medium, diluted 1/100 in M63 medium and grown until early exponential phase (OD_{600} = 0.1 - 0.2). The cells were harvested by centrifugation, washed twice with 1x M63 salts and resuspended in 1x M63 salts + Cm (25µg/ml) to an OD_{600} of 1.0. Aliquots of 1ml were kept on ice for up to two hours before use. Prior to use the cells were warmed to 37°C in a water bath. Doubling dilutions of microcin extracts were added to the cells giving dilutions from 1/4 to1/64, 2µl of ^{35}S-methionine/cysteine (PRO-MIX, 20mCi/ml) added and tubes incubated. MC4100 without microcin extract was used as the control. After 1, 5, 10 and 20 minutes a 100µl aliquot from each tube was removed, added to 900µl of TE and filtered through a 0.2µm nitrocellulose filter. The filters were washed in 1x M63 salts and pinned to a polystyrene board. After drying at 37°C, the filters were placed in a scintillation vial, 5ml of scintillation liquid added and the counts per minute (cpm) measured in a Wallac 1410 Liquid Scintillation Counter (Pharmacia).

A modification to this method was made by using a 1/8 dilution of Mcc24 extract and removing 102µl of culture at the various time points. A 2µl aliquot was diluted to 10^{-2}, 10^{-4} and 10^{-6} and 10µl from each dilution was plated on an LB plate to determine cell concentration. The cells in the remaining 100µl were pelleted by centrifugation and the supernatant transferred to a clean eppendorf tube with 1ml of scintillation fluid. The pellet was redissolved in 100µl TE, the cells harvested and

redissolved in 1ml of scintillation fluid. The cpm of the cells and supernatant was measured as described previously.

2.10.8 NUCLEASE ACTIVITY ASSAY (Pugsley and Oudega, 1987).

The Mcc24 extract was prepared (Methods 2.9.5) and the protein pellet dissolved in 1/1000th the original volume of phosphate buffer. A 2µl aliquot of the extract was mixed with 2µg of λ *Hin*dIII cut DNA or uncut pBR322 DNA and Reaction Buffer to give a final volume of 20µl. The mixture was incubated at 37°C and at various time points 4µl removed and mixed with 16µl of Dilution Buffer. The samples were immediately heated in a boiling water bath for two minutes and then placed on ice. At the end of the experiment the samples were analysed by agarose gel electrophoresis in a 0.6% agarose gel.

CHAPTER 3.

RESULTS.

3.1 ANALYSIS OF THE MICROCIN 24 ENCODING GENETIC REGION.

3.1.1 GENERATION OF MICROCIN 24 ENCODING DERIVATIVES.

Previously, a 25.14kb fragment from the Mcc24 encoding plasmid p24-2 had been cloned into pBR322 producing the recombinant plasmid pGOB34 (Figure 9). To further analyse the Mcc24 genes located on this fragment, it was first necessary to reduce the size of the DNA fragment encoding Mcc24 production , immunity and export. Derivatives of pGOB34 were generated and then tested for microcin production and immunity using both the patch and overlay tests (Methods 2.3.3; Figure 10). Digestion of pGOB34 with *Eco*RI or *Sal*I followed by self ligation generated the Mcc^- Imm^- clones pGOB341 and pGOB343 respectively. Linearised pGOB34 (*Hin*dIII cut) was subjected to partial digestion with *Eco*RI (2.5 units, Figure 11) and ligated to *Eco*RI digested pUC18 producing the clone pGOB342 (8.7 kb insert, Mcc^+). Re-ligation of *Eco*RI cut pGOB342 resulted in the 8.7 kb insert being ligated to pUC18 in the opposite orientation to that of pGOB342, producing pGOB420 as well as two other deletion derivatives pGOB421 (4.2kb insert, Mcc^- Imm^-) and pGOB423 (4.5kb insert, Mcc^- Imm^+). Digestion of pGOB420 with *Sal*I followed by re-ligation generated pGOB200 (Mcc^- Imm^+). The insert from pGOB421 was transferred to pACYC184 by digestion of both with *Eco*RI followed by ligation and the resulting recombinant plasmid named pLOB421.

In order to reduce the insert DNA size and because of the lack of suitable restriction sites for cloning, mini-Tn*10* was used to create extra restriction sites in pGOB420 without disrupting Mcc24 expression. The λ suppressing strain *E. coli* W3110 was first transformed with pGOB420 and then infected with λNK1316 as described (Methods 2.5.1). Transductants were selected on LB+Amp+Kan+Napp plates and their DNA electroporated into MC4100 to select for plasmid insertions. A total of 44 transformants were obtained and tested for microcin production using the patch test. Twenty four showed a microcin positive phenotype and 12 were selected for further

analysis. Restriction mapping identified four plasmids that contained the mini-Tn*10* insert in a suitable position to allow further cloning experiments. Utilisation of the extra *Bam*HI sites provided by mini-Tn*10* during to generate appropriate fragments for cloning, failed to produce a suitable Mcc24^{+} recombinant plasmid.

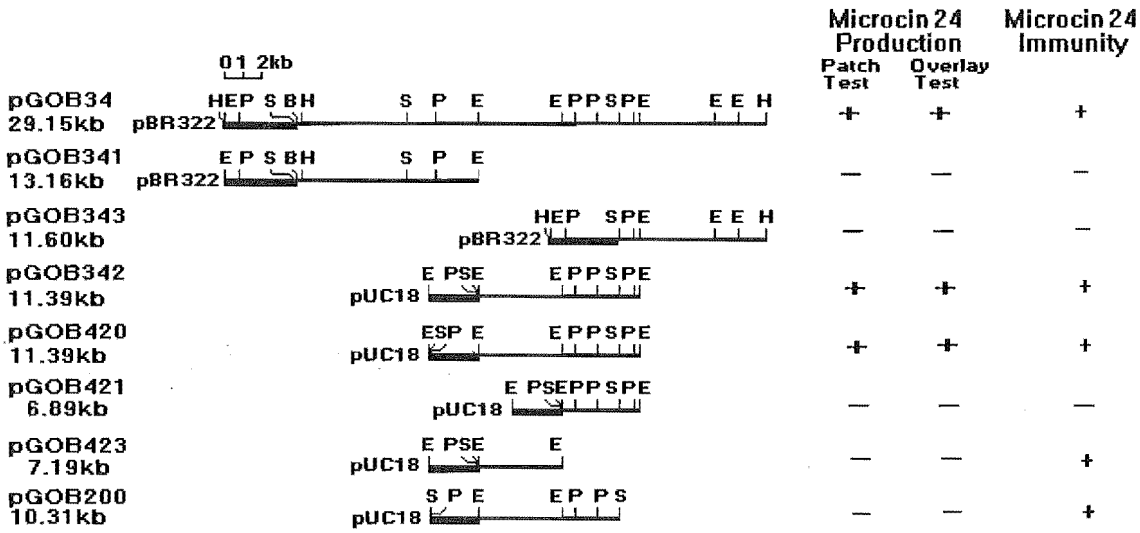


Figure 10. Restriction maps and phenotypes of pGOB34 derivatives. Derivatives of pGOB34 were tested for Mcc24 activity using the patch and overlay tests. Mcc24 immunity was determined using the patch test using recombinant plasmid-bearing *E. coli* strains as the lawn bacteria. Restriction enzymes B, *Bam*HI; E, *Eco*RI; H *Hin*dIII; P, *Pst*I; S, *Sal*I (Modified from O'Brien and Mahanty, 1994).

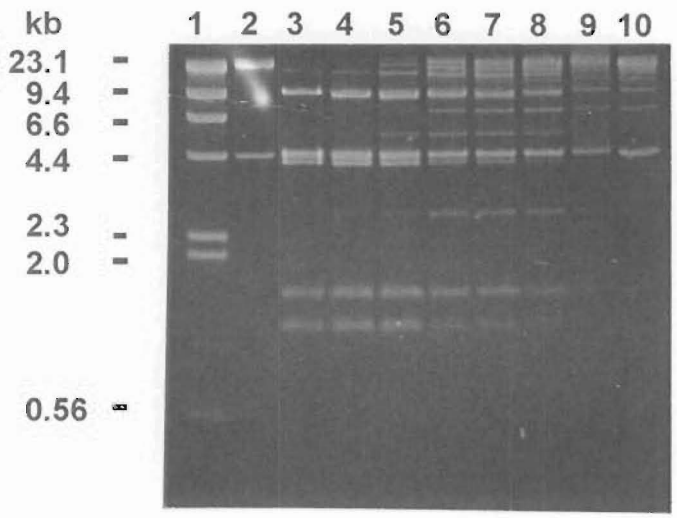


Figure 11. *Eco*RI partial digestion of linearised pGOB34. Partial digestions using *Eco*RI were performed after linearisation of pGOB34 by *Hin*dIII digestion. Gel electrophoresis using a 1.2% agarose gel was used to analyse the digestions. Lanes: 1, Lambda *Hin*dIII standard; 2, pGOB34 linearised by *Hin*dIII digestion; 3, pGOB34 *Hin*dIII/*Eco*RI complete digest; 4, *Eco*RI 5 units; 5, *Eco*RI 2.5 units; 6, *Eco*RI 1.67 units; 7, *Eco*RI 1.25 units; 8, *Eco*RI 0.83 units; 9, *Eco*RI 0.56 units; 10, *Eco*RI 0.28 units.

The second approach to reduce the Mcc24 encoding region was partial digestion of pGOB420 with *Sau*3AI (0.67 units) and ligation to *Bam*HI cut pBR322. After overnight incubation at 37°C, each transformation plate was covered in a lawn of cells, with small clearing zones occuring in the lawn. Using a binocular microscope, the bacterial colony at the centre of each clearing zone was collected, purified and Mcc24 activity confirmed using the patch test. Twenty Mcc24$^+$ strains were chosen from which the plasmid DNA was prepared and analysed by restriction analysis. A number of recombinant plasmids were identified (Figure 12A), of which pGOB18 was found to contain the smallest DNA insert and became the subject of further investigation. A deletion derivative of pGOB18 was generated by *Eco*RI digestion and religation of the DNA. The resulting plasmid, pGOB181, contained a 2.01kb insert and failed to produce microcin in the patch test but did show immunity to Mcc24 (Figure 12B).

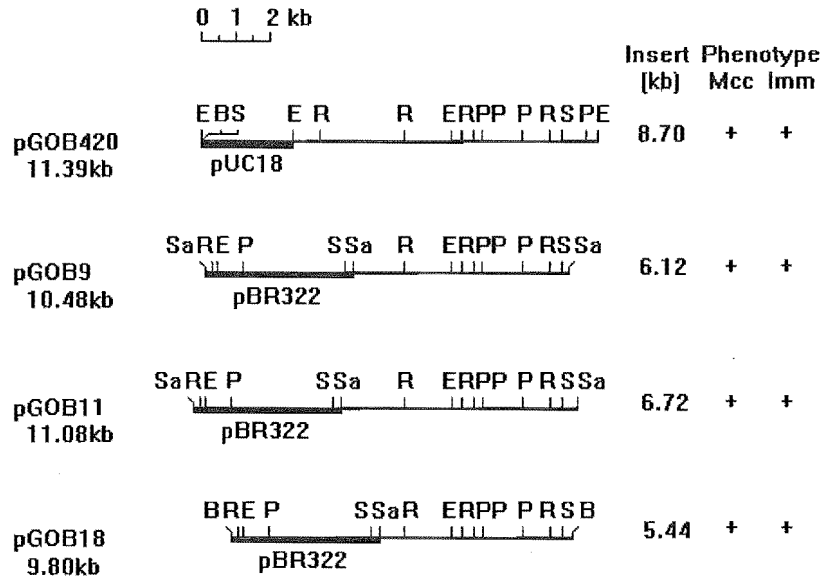
In order to determine the presence of complementation groups, as had been previously demonstrated in studies on ColV (Gilson *et al.*, 1987), an attempt was made to transfer the pGOB18 insert DNA into pACYC184. Partial digestion of pGOB18 DNA linearised by *Hind*III was performed using *Sau*3AI (0.11 units), followed by ligation to *Bam*HI cut pACYC184. Two recombinant plasmids, pLOB21 and pLOB8, were generated. *E. coli* MC4100 harbouring each derivative were determined to be deficient for Mcc24 production and immunity (Figure 12B). Because the constructs contained portions of the pGOB18 insert that may contain genes of interest, the constructs were stored for future use.

*3.1.2 MINI-Tn*10lac *KAN^R MUTAGENESIS OF pGOB18.*

Having established the restriction map and phenotype of pGOB18, the recombinant plasmid encoding the genes for Mcc24 expression, it was then necessary to determine the boundaries of the Mcc24 genes by mini-Tn*10* mutagenesis. Mini-Tn*10lacZ*'Kan^R was used because this would generate translational fusions, allowing the position of the Mcc24 promoter(s) and the direction of transcription to be determined. MC4100(pGOB18) was first transformed with pNK2882 to provide the transposase function *in trans*. Mutagenesis using λ NK1205 was performed as described (Methods 2.5.2) and the plasmid DNA from the transductants was then electroporated into MC4100 and analysed for plasmid insertions. A total of 91 transformants were obtained and then screened for tetracycline sensitivity, indicating the loss of pNK2882, β-galactosidase activity and Mcc24 production. A total of 12 dark blue Mcc24$^-$, 8 light blue Mcc24$^-$ and 6 Mcc24^{RED} (both blue and white) were chosen and their insert position was mapped (Figure 13A). The level of β-galactosidase activity was determined for all of the mutants. Results showe that the activity from the light blue mutants (transcribing right to left) was negligible (Figure 13B). This indicated that only transcription from left to right (Figure 13A) generated active β-galactosidase fusions which suggested the presence of two or three promoters, all transcribing from left to right.

Chloroform was used to identify insertions in transport genes as observed previously with ColV (Gilson, *et al.*, 1987). Treatment of Mcc24$^-$ mutants with chloroform using the overlay test failed to show microcin activity.

A.



B.

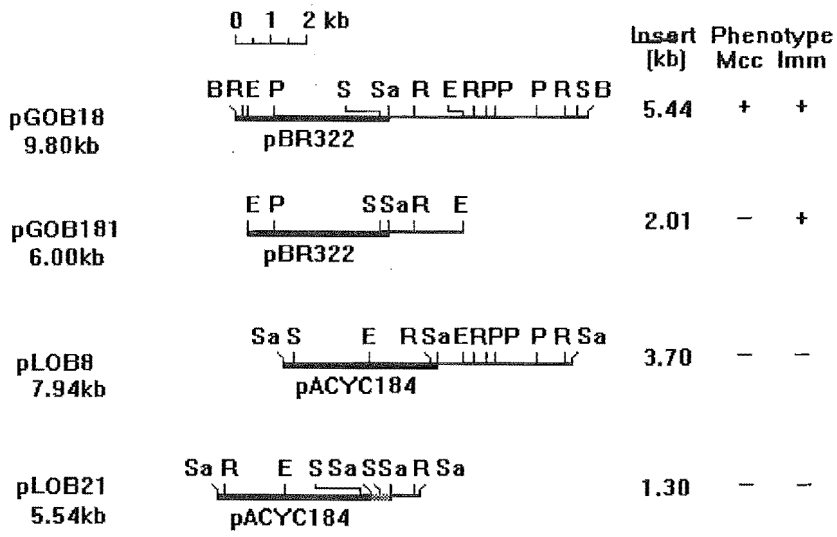
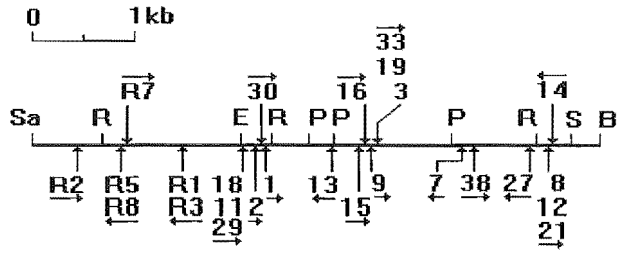


Figure 12. Further derivatives encompassing the microcin 24 encoding region. **A.** Derivatives of pGOB420 generated by *Sau*3AI partial digestion. **B.** Recombinant plasmids derived from pGOB18. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; R, *Eco*RV; P, *Pst*I; S, *Sal*I; Sa, *Sau*3AI.

A.



B.

Mutant	Microcin production (mm)	β-gal activity (Miller units)	Mutant	Microcin production (mm)	β-gal activity (Miller units)
1	-	28.75	19	-	1.34
2	-	21.10	21	-	94.17
3	-	1.67	27	-	1.74
7	-	1.33	29	-	683.56
8	-	109.7	30	-	25.52
9	-	74.74	33	-	23.02
11	-	650.50	38	-	36.72
12	-	53.84	R1	2	0.47
13	-	0.67	R2	3	49.28
14	-	2.37	R3	2	0.57
15	-	40.46	R5	1	0.03
16	-	14.36	R7	0.5	1.69
18	-	655.05	R8	1	0.02

- denotes microcin minus.

Figure 13. Mutagenesis of pGOB18 using mini-Tn*10lac*. A. Location of mini-Tn*10lacZ*' insertions within pGOB18. Vertical arrows denote position of insert and horizontal arrows denote the direction of transcription of LacZ. All mutants were Mcc24$^-$ except R_1-R_8 (R, Mcc24^{RED}) Key: B, *Bam*HI; E, *Eco*RI, P, *Pst*I; R, *Eco*RV; S, *Sal*I; Sa, *Sau* 3AI. **B.** Microcin 24 production and β-galactosidase activity of pGOB18::mini-Tn*10lac* mutants. Mutants with a reduced microcin phenotype are designated "R". Each sample was tested twice with two readings per test. The average OD_{420}, OD_{550} and OD_{600} was used to calculate the activity of β-galactosidase (Miller units). Full activity was defined as 1000 Miller units (Miller, 1972).

Before determining the complete DNA sequence of the pGOB18 insert DNA, it was first necessary to obtain some information on the genes involved in Mcc24 production. Utilising the primer MKBGAL1, which binds within the first 120bp of *lacZ*, it was possible to derive DNA sequence from the end of the pGOB18::mini-Tn*10* insertions #9, 14, 18 and R7. These insertions were chosen because they represented the entire Mcc24 encoding region. DNA sequence was obtained by the Center for Gene Technology (University of Otago) and sent to the Blast Server in order to identify similarities to other DNA and protein sequences in the data bank. DNA and protein sequence similarity to regions of the ColV transporter CvaA was found in insertions #18 and 9, and to regions of the ColV transporter CvaB in insertion #14. Insertion #R7 did not show any significant sequence similarity to known bacteriocins or bacteriocin related genes (Figure 14).

*3.1.3 TRANS-COMPLEMENTATION BETWEEN pGOB18::MINI-Tn*10lac *MUTANTS AND THE COLICIN V TRANSPORT GENES.*

DNA sequence analysis had identified a degree of sequence similarity between the ColV transporters CvaAB and regions encoded by pGOB18, therefore trans-complementation was used to determine whether transport functions encoded by pGOB18 were homologous to CvaAB. The pBR322 compatible pACYC184 derived plasmids pHK22-6(*cvaAB*$^+$ *cvaC*$^-$) and pLY21(*cvaAB*$^-$ *cvaC*$^+$) were transformed into *E. coli* MC4100 harbouring either pGOB18::mini-Tn*10lac* #9, 14, 18 or R7, and transformants spread on two LB+Amp+Cm+Kan plates to select for the complementing plasmids. Fifty transformants from each were screened for microcin expression by complementation using the patch and overlay tests (Figure 15) and the plasmid profiles of representatives checked by gel electrophoresis (Data not shown). Expression of Mcc24 occurred when insertions #9,18 and 14 were complemented with pHK22-6, identifying these insertions as being in transport genes. Complementation between insertion #R7/pHK22-6 and #R7/pLY21 produced a slightly increased microcin halo (1mm) and a very clear, 1.5mm halo respectively compared to #R7 alone, suggesting that ColV might be expressed by #R7/pLY21.

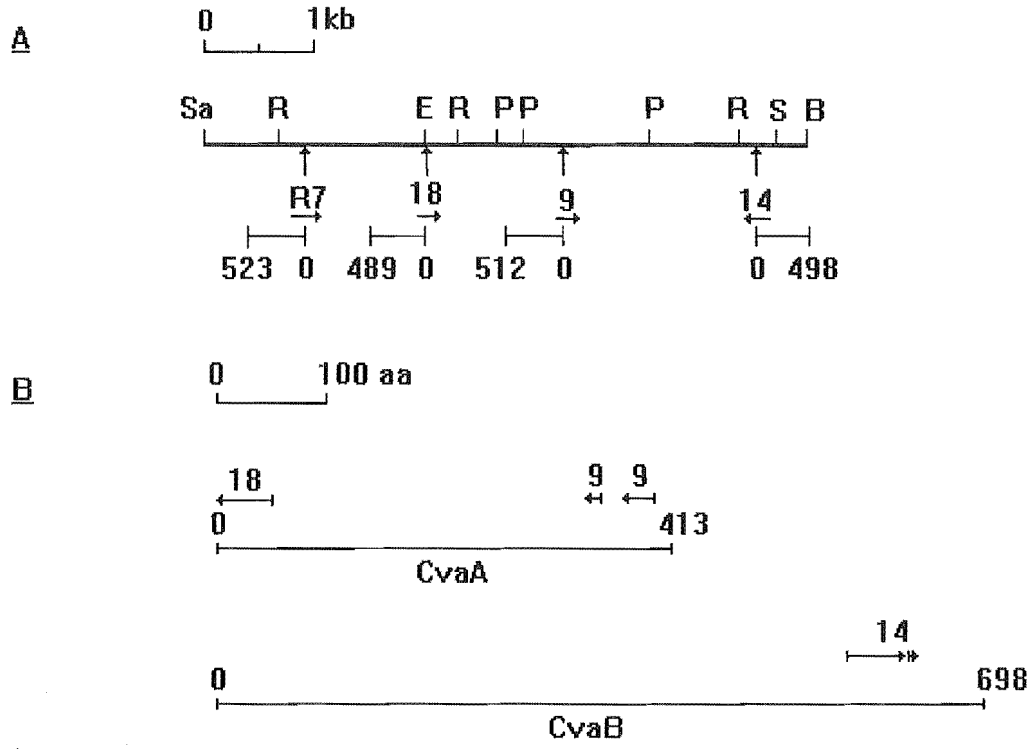


Figure 14. DNA sequence analysis of pGOB18::mini-Tn*10lac* mutants. A. DNA sequence from mini-Tn*10lacZ'* mutants. Vertical arrows indicate the position of insertion, horizontal arrows indicate the direction of transcription of *lacZ*. The number of bases sequenced from each mutation are indicated. Enzymes: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Rv, *Eco*RV, S, *Sal*I; Sa, *Sau*3A I. **B.** Amino acid sequence similarities. The DNA sequences were compared to known sequences using the Blast server. Amino acids (aa) 4-153 from mutant #18, aa 1-84 and aa 147-188 from mutant #9 showed 68% identity with aa 1-50, 71% identity with aa 370-397 and 68% identity with aa 336-349 of CvaA respectively. Amino acids 1-162 and aa 167-169 from mutant #14 showed 79% identity with aa 574-627 and 88% identity with aa 629-637 of CvaB respectively. Mutant R_7 did not show any significant protein similarity to known microcins. Horizontal arrows indicate the direction of amino acid reading from mutants.

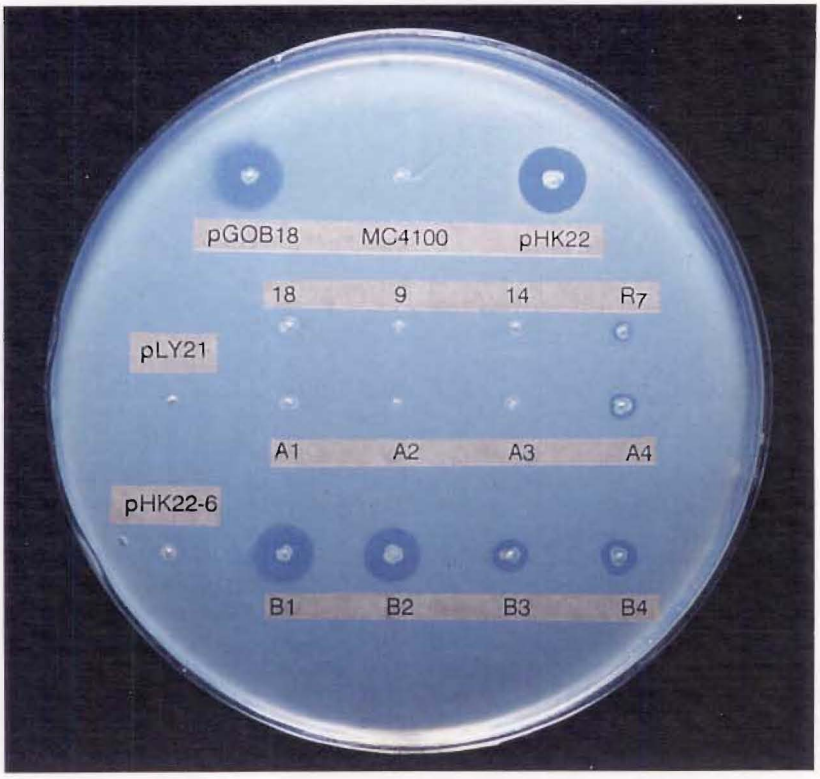


Figure 15. Trans-complementation between pGOB18 insertions and colicin V transport proteins. Complementation was assayed using the patch test on a lawn of *E. coli* MC4100. All plasmids were harboured within *E. coli* MC4100. Plasmids: pGOB18::mini-Tn*10lac* #18, 9, 14 and R7; pLY21 (*cvaC*$^+$); pHK22-6(*cvaAB*$^+$); A1-A4, complementation between pLY21/pGOB18::mini-Tn*10lac* #18, 9, 14 and R7; B1-B4, complementation between pHK22-6/pGOB18::mini-Tn*10lac* #18, 9, 14 and R7. Controls: pGOB18 (Mcc24$^+$); pHK22(ColV$^+$); MC4100.

Complementation between insertions #9, 14, 18 and R7 with pYIE10, a plasmid containing the transporter for *Serratia entomophila* chitinase, was also attempted because pYIE10 had previously been shown to complement CvaAB$^-$ mutants (Shum, 1992). All transformants failed to show any complementation. Complementation between pGOB181 (Results 3.1.1) and pYIE10 also failed to produce Mcc24 indicating that pYIE10 could not complement the transport functions absent in pGOB181.

3.1.4 ANALYSIS OF pGOB18 ENCODED PROTEINS USING MINICELLS.

Minicells are small anucleate cells produced by specific strains of *E. coli*. These strains contain mutations in the *min* and *mtl* genes resulting in unequal division of the cell during logarithmic phase growth and the budding off of the small nonviable minicells which can be separated from normal cells using density gradient centrifugation. If the parental strain carries a plasmid, some plasmid molecules may segregate into the minicells during cell division. Since minicells contain all the RNA and protein required for transcription and translation, incubation with ^{35}S-labelled amino acids (methionine) results in the identification of proteins arising from the plasmid encoded genes (Adler *et al.*, 1967, Meagher *et al.*, 1977).

E.coli P678-54T was chosen for the generation of minicells from plasmid containing parental cells. The plasmids to be analysed were first electroporated into P678-54T and the presence of the plasmid checked by DNA extraction and restriction analysis. Minicells were prepared from strains carrying pBR322 (control), pGOB18 and various pGOB18::mini-Tn*10lac* insertions #18, 9, 7, 21, 14 (Mcc24$^-$), R7 and R2 (Mcc24^{RED}). At this point in the research it was thought that Mcc24 was in fact a colicin so SDS-PAGE was performed to maximise proteins from 20-90 kDa (Figure 16). Protein bands were identified with approximate sizes 47kDa, 42kDa, 40kDa and 19.5kDa.

3.1.5 DEFINING THE LOCATION OF THE MICROCIN 24 GENES BY COMPLEMENTATION.

In contrast to previous complementation experiments where various pGOB18::mini-Tn*10*, Mcc24$^-$ insertions were used, plasmid derivatives were complemented with pLY21 (*cvaC*$^+$)and pHK22-6 (*cvaAB*$^+$) in order identify the location of the Mcc24 genes. Both pHK22-6 and pLY21 were transformed into 71-18(pGOB421) and 71-18(pGOB423) with transformants selected on LB+Amp+Cm plates and tested for microcin production using the patch test. Positive complementation was observed only between pGOB423/pHK22-6 indicating that the structural and immunity region was encoded by pGOB423 (Figure 17).

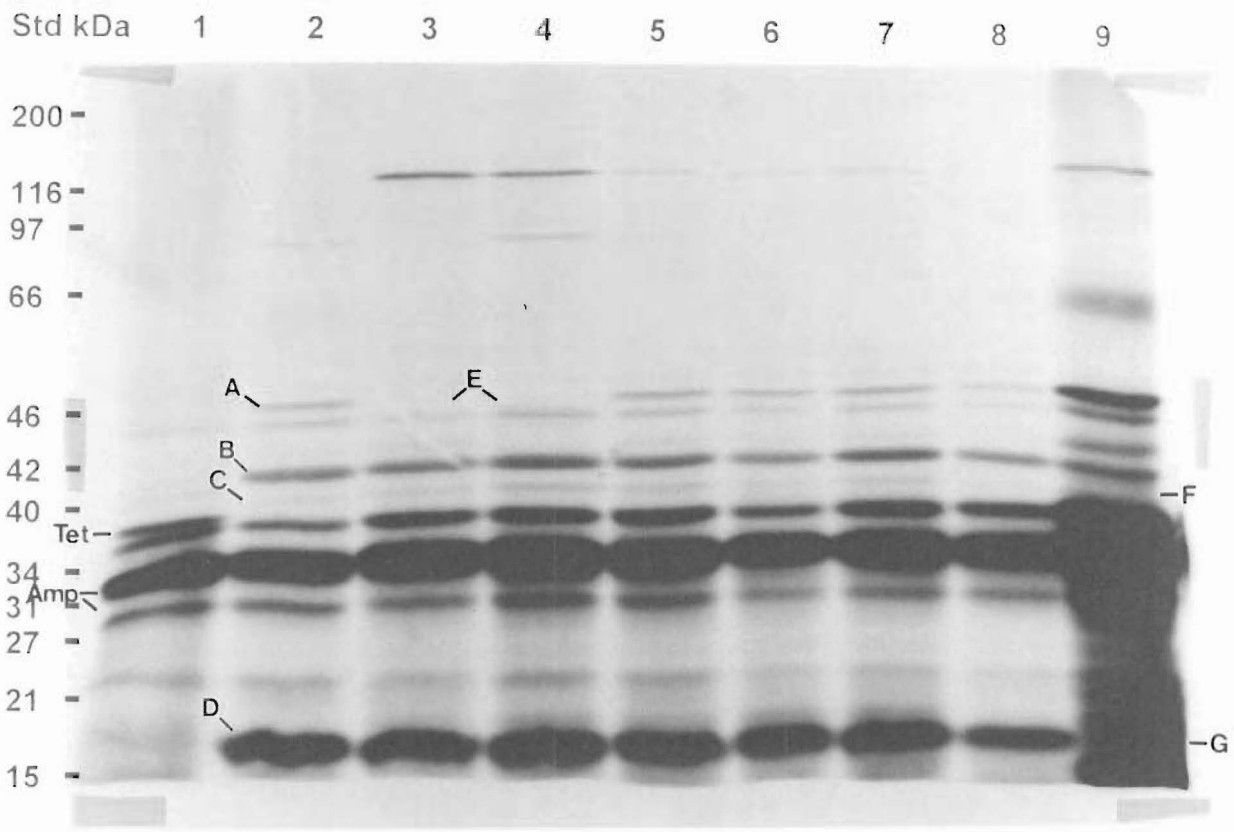


Figure 16. Analysis if pGOB18 encoded proteins using minicells. Plasmids were electroporated into *E.coli* P678-54T and minicells separated from normal cells on a 20% sucrose gradient by centrifugation. Plasmid encoded proteins were labelled with ^{35}S-methionine/cysteine mix (Promix, Amersham) and analysed by SDS-PAGE on a 10% acrylamide gel. Lanes: 1, pBR322; 2, pGOB18; 3-9, pGOB18::mini-Tn*10lac* insertions #18, #9, #7, #21, #14, #R7 and #R2 respectively. Protein bands: A, 47kDa; B, 42kDa; C, 40kDa; D, 19.5kDa; E-G, absence of 47kDa, 40kDa and 19.5kDa proteins respectively. Amp, β-lactamase 31kDa and 28kDa; Tet, Tet^R protein 37kDa (Sancar *et al.*, 1979).

To further define gene boundaries the derivative pGOB181 was complemented with pHK22-6 and pLOB423 (Results 3.1.1), however complementation was only found between pGOB181/pHK22-6. This result indicated that pGOB181 encoded the Mcc24 structural gene and that the *Eco*RI site located between pGOB181 and pLOB421 was important for Mcc 24 expression. When MC4100(pGOB181) was used as a bacterial lawn in the patch test, pGOB18 failed to produce a microcin halo showing that pGOB181 also encoded the Mcc24 immunity gene.

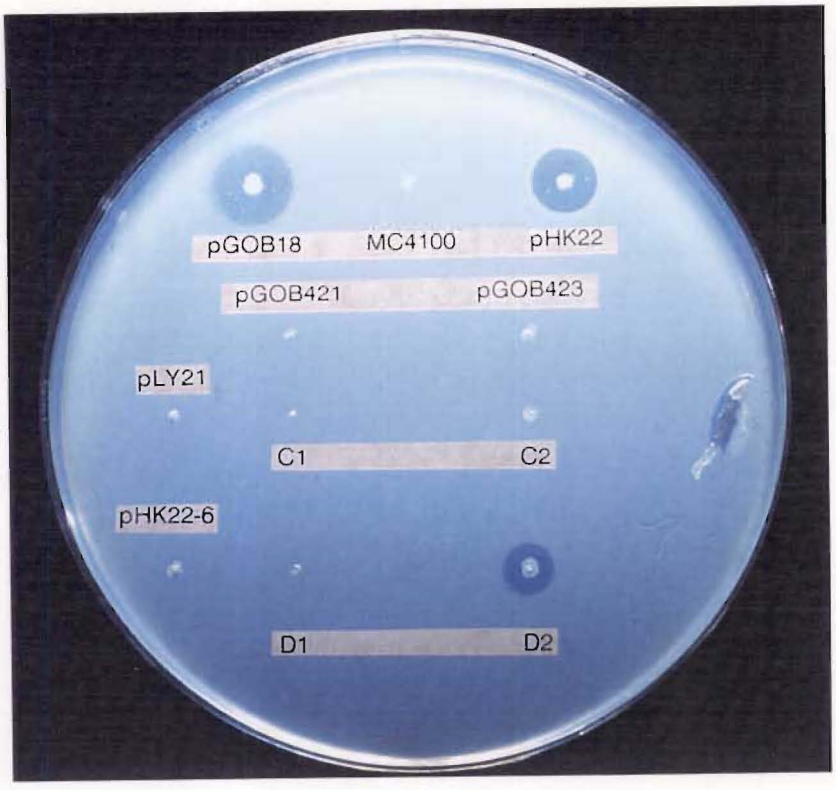


Figure 17. Trans-complementation between microcin 24 and colicin V genes. *E.coli* 71-18(pGOB421) and 71-18(pGOB423) were transformed with pHK22-6 and pLY21. Transformants were screened for microcin production using the patch test. All plasmids were harboured within *E. coli* MC4100. Plasmids: pGOB421(Mcc24$^-$); pGOB423(Mcc24$^-$); pLY21(*cvaC*$^+$); pHK22-6(*cvaAB*$^+$); C1 and C2, pLY21/pGOB421 or pGOB423 respectively; D1 and D2, pHK22-6/pGOB421 or pGOB423 respectively. Controls: pGOB18(Mcc24$^+$); MC4100; pHK22(ColV$^+$).

Previous DNA sequencing analysis and complementation results had indicated the presence of two transport genes, therefore the gene boundaries were defined by complementation between pLOB8, a subclone of pGOB18 (Results 3.1.1) and various pGOB18::mini-Tn*10lac* insertions. Plasmid DNA of pLOB8 was transformed into MC4100 harbouring the pGOB18::mini-Tn10 plasmids #7, 9, 14, 18, 33 and R1. Complementation was only seen between #18/pLOB8 and #9/pLOB8 resulting in halos of 2mm and 1mm respectively, confirming that mutations 18 and 9 are in the same gene as indicated by the sequence data. The other mutations are in a second transport gene, the start of which lies in the 50bp between mutations 9 and 33 (Figure 18).

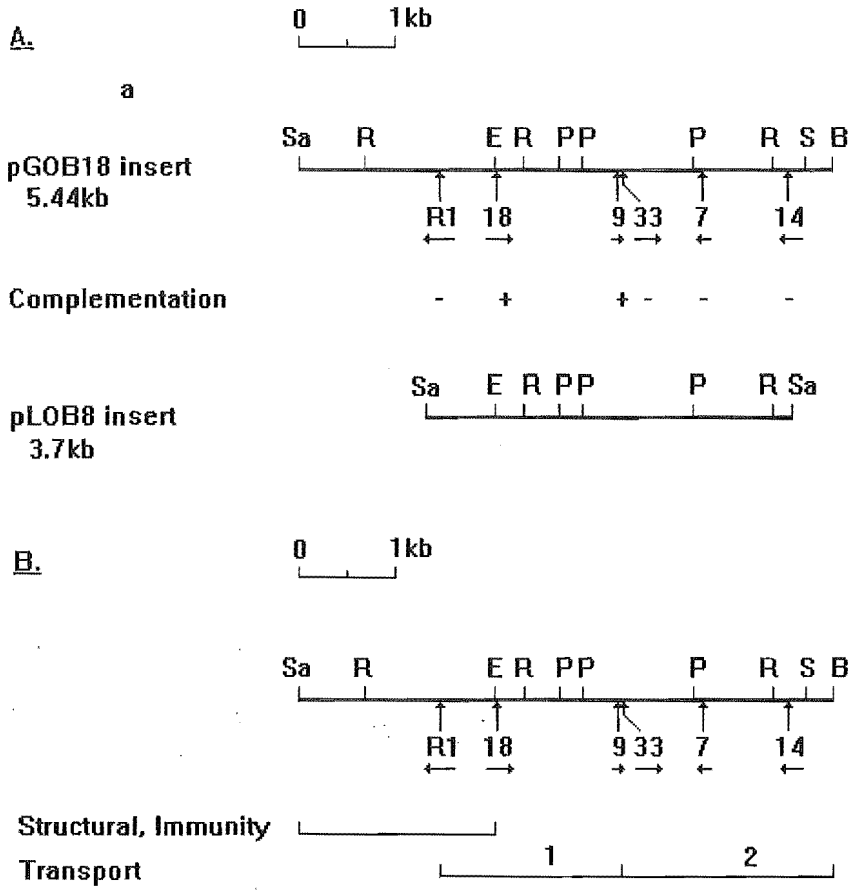


Figure 18. Defining microcin 24 gene boundaries by trans-complementation. A. Complementation between pLOB8 and pGOB18::mini-Tn*10lac* mutants. Complementation *in trans* (+) was indicated by the production of a microcin halo. No complementation (-) was indicated by no microcin halo. **B.** Location of the Mcc24 genes as indicated by complementation. Key: B, *Bam*HI; E, *Eco*RI; R, *Eco*RV; P, *Pst*I; S, *Sal*I; Sa, *Sau*3AI.

3.1.6 RECIPROCAL COMPLEMENTATION BETWEEN MICROCIN 24 AND COLICIN V TRANSPORT GENES.

The ability of the ColV transporters CvaA and CvaB to export Mcc24 had been previously shown (Results 3.1.3). In order to determine if this complementation could be reciprocated, resulting in the export of ColV, pGOB18, pGOB181 and pGOB18::mini-Tn*10lac* mutant R7 were transformed with pHK22-6 and pLY21 and bacteriocin production tested using the patch test. ColV activity was tested on lawns of

MC4100, MC4100(pGOB18) and MC4100(pHK11) in order to distinguish between production of Mcc24 and ColV, since MC4100(pGOB18) would be resistant to Mcc24. The export of ColV by the Mcc24 transport system was confirmed since a ColV halo was produced by pGOB18/pLY21 and R7/pLY21 on a MC4100(pGOB18) lawn (Figure 19).

3.2 DIDEOXYNUCLEOTIDE DNA SEQUENCING.

3.2.1 CLONING INTO pDELTA1 AND pBLUESCRIPT.

To facilitate the nucleotide sequencing of the Mcc24 genes it was necessary to create overlapping nested deletions of the Mcc 24 encoding region using the Deletion Factory™ System (BRL Life Technologies). The Mcc24 genetic region was cloned into pDELTA1, the plasmid used by this nested deletion system, and several constructs generated in order to create nested deletions covering the entire insert of pGOB18 (Figure 20; Appendix 4).

Cloning into pDELTA1 was unsuccessful at first, however the Mcc24 encoding region was sucessfully cloned by digesting pGOB18 and pDELTA1 with *Bam*HI and *Eco*RI, followed by ligation and electroporation into *E. coli* DH10B. A total of 600 transformants were obtained of which 350 were white, indicating ligation into the *lacZ* polylinker. A total of 34 were found to produced microcin halos and the plasmids from 10 colonies were analysed by gel electrophoresis. All plasmids contained the same fragment and of these, pGΔ185, containing all of pGOB18 cloned into pDELTA1, was chosen for further experiments.

A deletion derivative containing the 3.4 kb fragment encoding most of the transport genes was generated from pGΔ185 by complete digestion of pGΔ185 with *Eco*RI followed by self ligation. The resulting derivative, pGΔ851, was then used to create nested deletions.

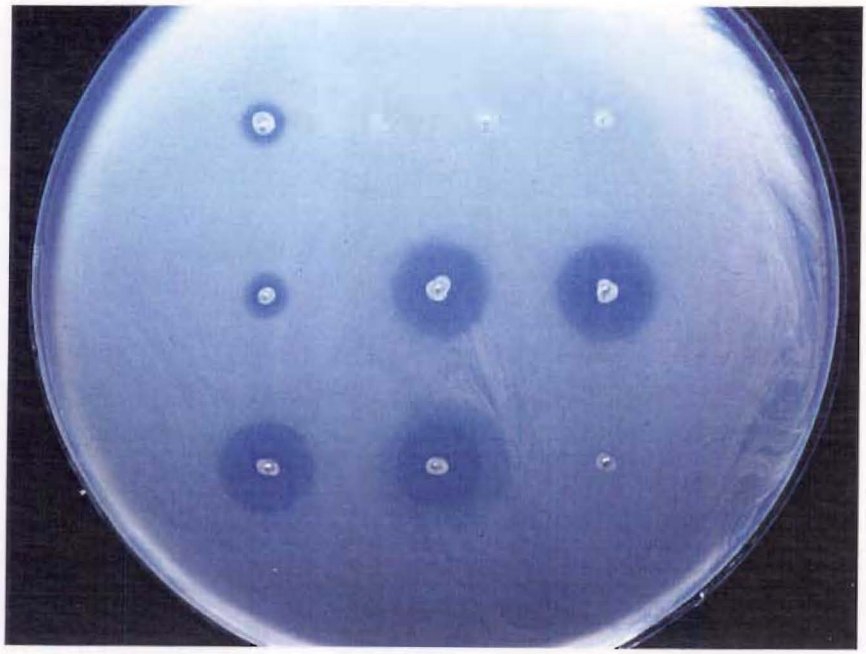
Transfer of the structural and immunity region (1.9 kb) from pGOB181 to pBluescript SK by *Eco*RI and *Sal*I digestion followed by ligation, produced the derivative pGSK813. The 1.9 kb fragment from pGSK813 was then cloned into pDELTA1 generating pGΔ813.

Figure 19. Reciprocal complementation between microcin 24 and colicin V transport proteins. MC4100(pGOB18), MC4100(pGOB181) and MC4100(pGOB18::mini-Tn*10lac* #R7) were transformed with pHK22-6 and pLY21. Tranformants were assayed for microcin production using the patch test. **A.** MC4100 lawn, control. **B.** MC4100(pGOB18) lawn, selection for ColV production. **C.** MC4100(pHK11) lawn, selection for Mcc24 production.

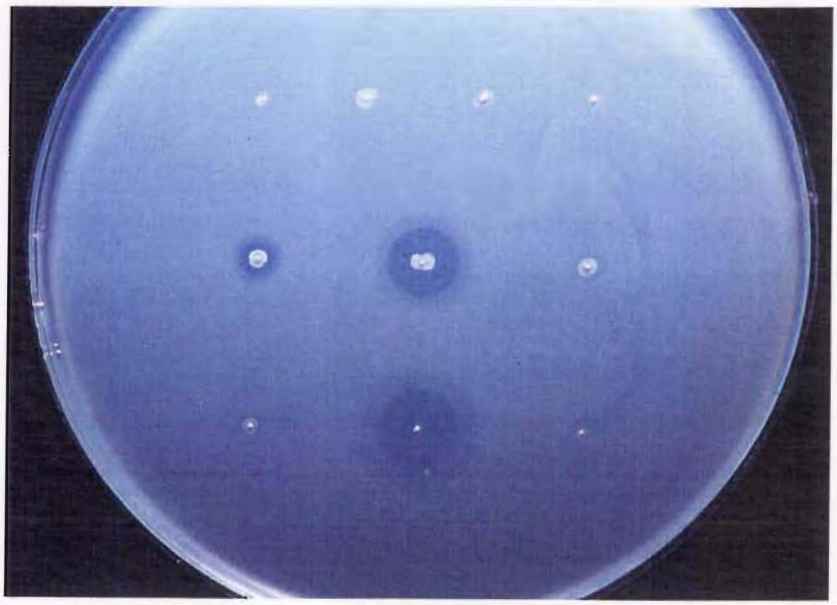
Plasmids:

pGOB18::mini-Tn*10lac* #R7	pGOB181	pLY21	pHK22-6
R7/pLY21	pGOB18/pLY21		pGOB181/pHK22-6
pGOB18	pHK11		MC4100

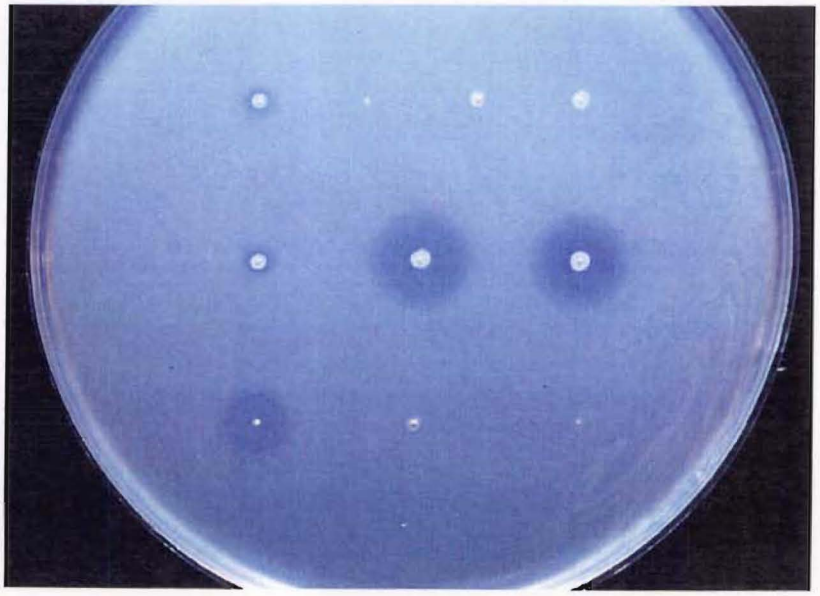
A.



B.



C.



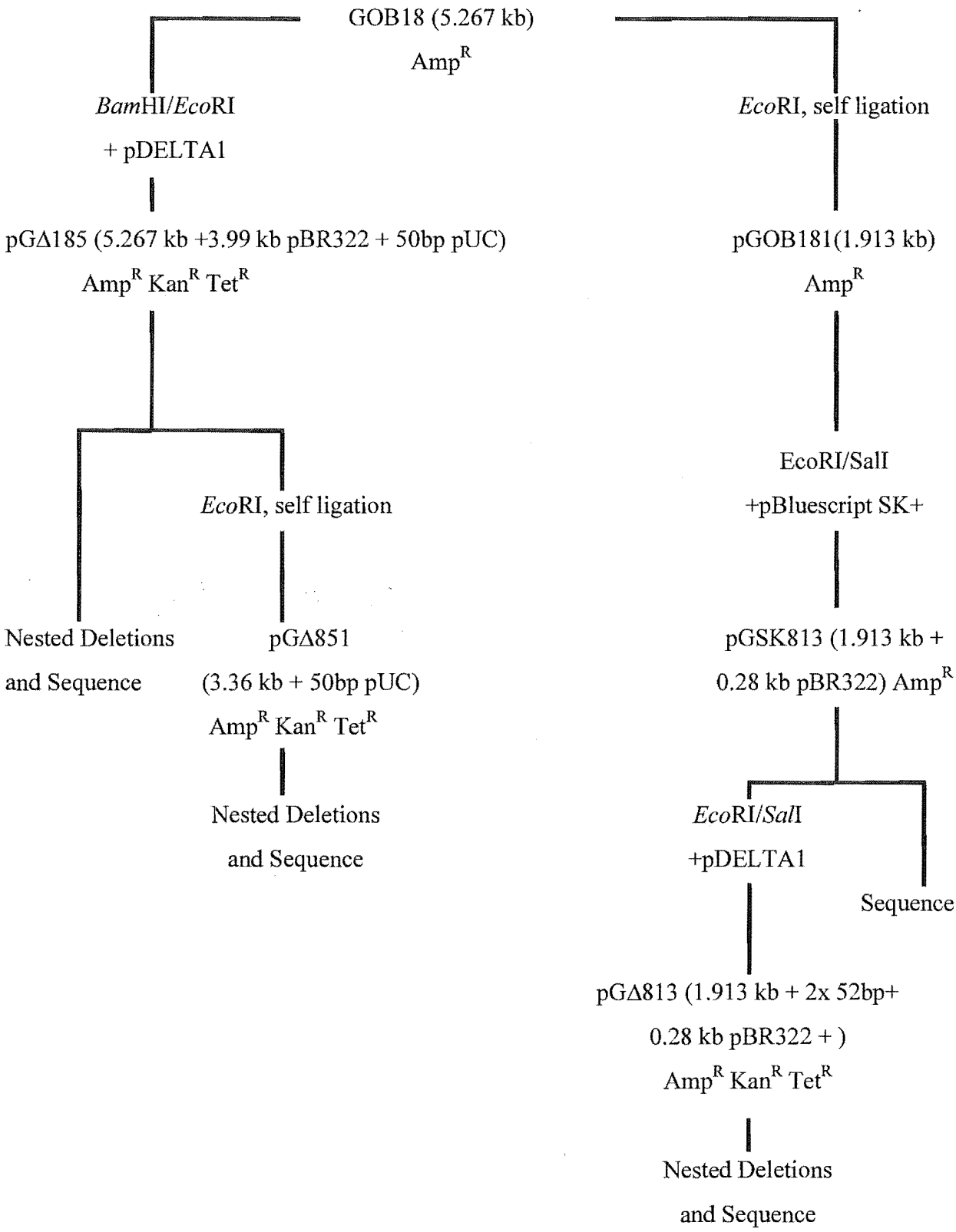


Figure 20. Generation of plasmids for DNA Sequencing. The size of each insert DNA is indicated in the brackets and vector sizes are not given. Antibiotic resistant markers are derived from vector molecules. For restriction maps of plasmids see Appendix 4.

3.2.2 CREATION OF NESTED DELETIONS AND NUCLEOTIDE SEQUENCING.

All the sequences generated by nested deletions or primer walking were aligned manually (Figure 21).

Transport region.

Nested deletions were generated from pDELTA1 as described in Methods 2.4.16. Nested deletions of pGΔ185 were selected by Kan^R and $Strep^R$, giving deletions which would allow sequencing of the sense strand as defined by mini-Tn*10lac* mutagenesis (Results 3.1.2). A total of 110 separate transformants were tested for microcin production and the plasmid from each transformant analysed by gel electrophoresis after digestion with *Hin*dIII. A total of 33 deletions were selected between 4.5-10.5 kb and sequenced using the T7 sequencing primer (Methods 2.6). Alignment showed that there were 16 individual sequences which spanned 2848 bp of the transport region (Figure 21), with the remainder being identical deletions or progeny.

To complete the sequence of the 3.36 kb transport region nested deletions were generated from pGΔ851 in both directions. A total of 62 $Kan^R/Strep^R$ clones (complementary strand) were screened for sizes between 5.2 - 8.7 kb and 14 plasmids were chosen for sequencing using the T7 primer with seven clones sequenced manually and seven sent to a commercial facility (Centre for Gene Research, Otago University). Deletions using Tet^R/Suc^R (sense strand) were screened for sizes between 4.9 - 8.3 kb. A total of 56 clones were screened with 14 chosen for sequencing at a commercial facility using the Sp6 primer.

Structural and immunity region.

Nested deletions of the structural and immunity region were generated in both directions from pGΔ813. A total of 90 clones were selected by Tet^R/Suc^R (coding strand) and screened by *Bgl*II digestion and selection 4.9 - 7.3 kb (Figure 22). Nineteen clones were sequenced using the Sp6 primer and the T7 polymerase sequencing kit (Methods 2.6; Figures 21 and 23). Nested deletions of the complementary strand ($Kan^R/Strep^R$) were selected for sizes between 5.6 - 7.7 kb after digestion with *Bgl*II and 11 out of 99 chosen for sequencing at a commercial facility.

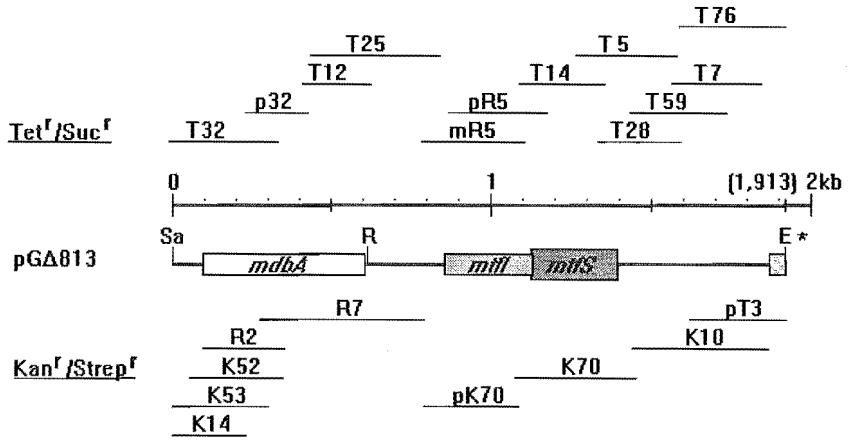
Primer walking.

In order to fill gaps in the sequence generated by the nested deletions, several pGOB18::mini-Tn*10lac* mutants (Results 3.1.2) were sequenced using the primers MKBGAL1 and mini-Tn*10lacZ'* (Appendix 3), and the insert positions presented in Table 5. A series of seven primers were also created from various nested deletion clones (Appendix 3) and used to prime sequence reactions.

Table 5. Insertion position of mini-Tn*10* in pGOB18::mini-Tn*10* mutants.

Mutant	Primer	Bases inserted between (5',3')
7	MKBGAL1	(3930,3931)
8	MKBGAL1	(4748,4749)
9	MKBGAL1	(3063,3064)
13	MKBGAL1	(2944,2945)
14	MKBGAL1 and mini-Tn*10lacZ'*	(4832,4833)
18	MKBGAL1	(2027,2028)
38	MKBGAL1	(4052,4053)
R2	MKBGAL1	(352,353)
R3	mini-Tn*10lacZ'*	(1447,1448)
R5	MKBGAL1	(785,786)
R7	MKBGAL1	(794,795)

A.



B.

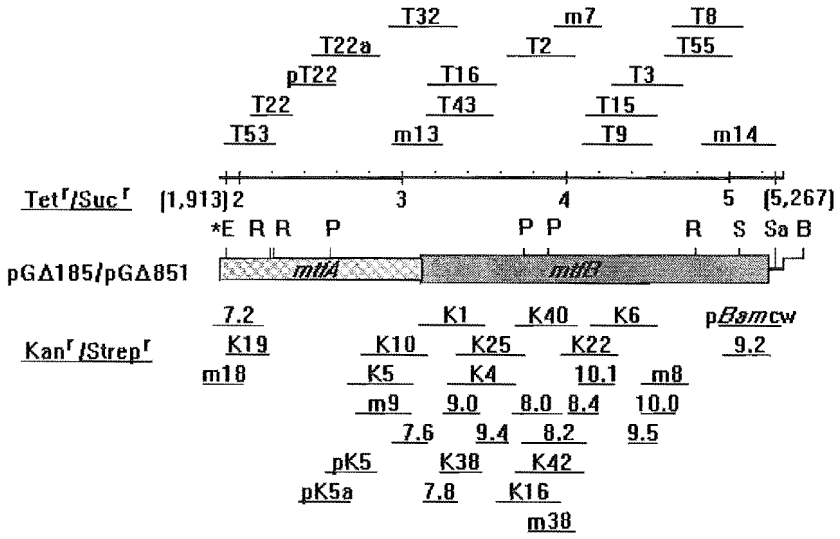


Figure 21. Alignment of DNA sequences obtained from nested deletions and primer walking. A. Sequence alignment of structural and immunity region using nested deletions of pGΔ813. **B.** Sequence alignment of transport region using nested deletion of pGΔ185. DNA sequences were obtained using various methods: p, primer walking; T, DNA sequencing using Tet^R deletions; K, DNA sequencing using Kan^R deletions; m, DNA sequencing using pGOB18::mini-Tn*10* insertions. Genes are indicated in shaded boxes and the number of nucleotides sequenced is indicated by a line. Restriction enzymes: P, *Pst*I; E,*Eco*RI; R, *Eco*RV; S, *Sal*I; Sa, *Sau*3AI.

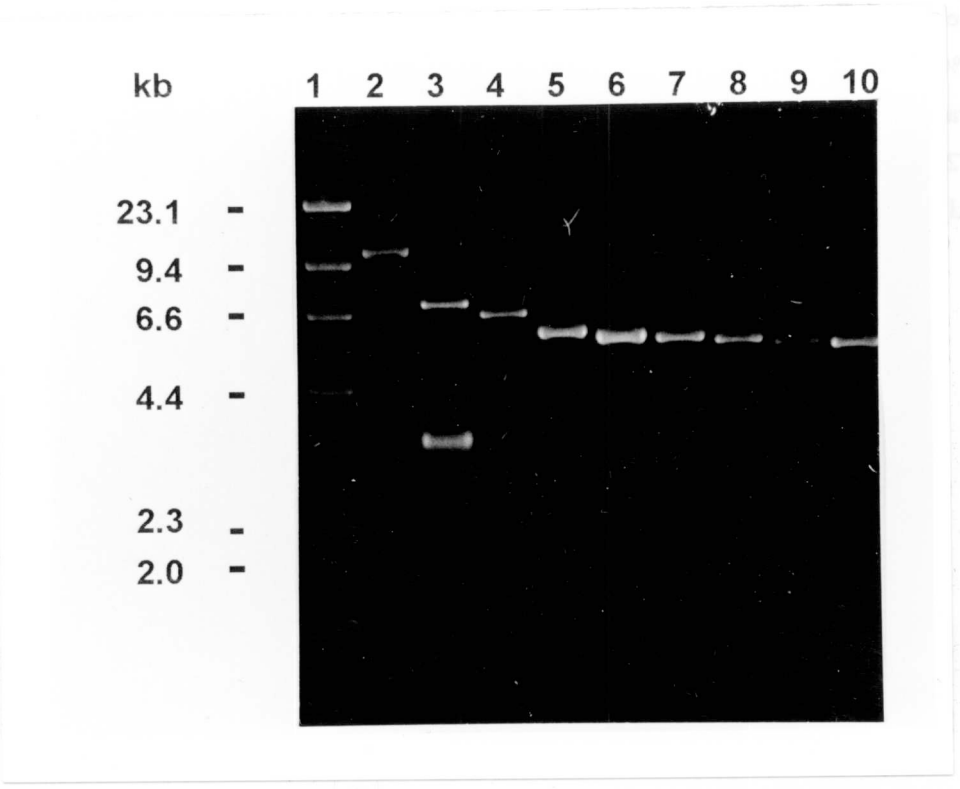
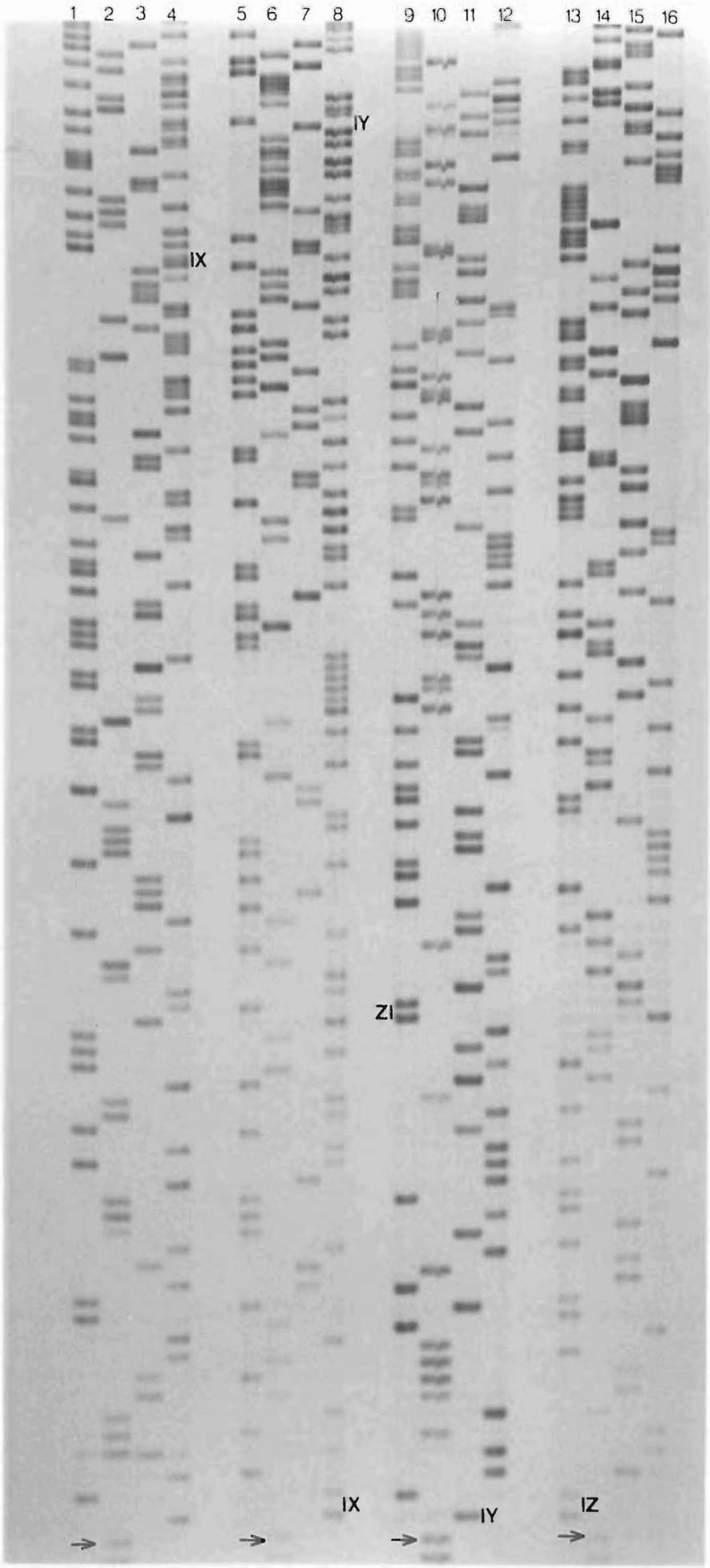


Figure 22. Agarose gel electrophoresis of nested deletions. Nested deletions of pGΔ813 were made using the Deletion Factory™ System (BRL, Life Technologies) and analysed in a 1.0% agarose gel after digestion with *Bgl*II. Lanes: 1, λ *Hin*dIII standard; 2, pGΔ813; 3, Deletion T32; 4, Deletion T25; 5, Deletion T14; 6, Deletion T5; 7, Deletion T28; 8, Deletion T59; 9, deletion T7; 10, Deletion T76.

Figure 23. Overlapping DNA sequences generated by pGΔ813 Tet^R/Suc^R nested deletions. Nested deletions of pGΔ813 were made using the Deletion Factory™ System (BRL, Life Technologies). Sequencing was performed using the T7 polymerase sequencing kit (Pharmacia) and fragments run on a 6% acrylamide gel using the LKB 2010 Macrophor Electrophoresis Unit. Arrows indicate the γδ transposon end 5'-AAACCCC-3', where the 3'-C is juxtaposed to new sequence. Lanes: 1-4 Deletion T28 A C G T; 5-8 Deletion T59 A C G T; 9-12, Deletion T7 A C G T; 13-16, Deletion T76 A C G T. Alignment: X, T28→T59; Y, T59→T7; Z, T7→T76.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
IY
IX
ZI
IX
IY
IZ

3.2.3 ANALYSIS OF DNA SEQUENCE.

The full nucleotide sequence of 5267 bp is shown in Figure 24. During sequencing it was found that pGOB18 contained an extra piece of DNA from the pUC18 *amp* gene at the 3'-OH end resulting in the retention of the *Bam*HI site (Appendix 4). This piece of DNA was obtained from *Sau*3A digestion of pGOB420 (Results 3.1.1). The clone pGΔ813 was found to contain a duplication of the *Eco*RI-*Sal*I polylinker fragment from pDELTA1 at the 5' end (Appendix 4). Analysis of the sequence using DNASIS identified five open reading frames which have been called *mdbA* (Mcc24 DNA binding); *mtfI* (Mcc24 immunity, 279bp); *mtfS* (Mcc24 structural, 270bp); *mtfA* (Mcc24 transporter A, 1242bp); *mtfB* (Mcc24 transporter B, 2121bp). Three operons have been identified, *mdbA*, *mtfI/mtfS* and *mtfA/mtfB*. The complete physical map of the pGOB18 insert DNA coding for Mcc24 expression is presented in Figure 25. DNASIS was also used to determine restriction enzyme sites (Appendix 5) and to translate the open reading frames determined by sequencing.

3.2.4 SEQUENCE SIMILARITY COMPARISONS.

In order to generate sequence comparisons between the proteins encoded by the nucleotide sequence presented in Figure 24 and known protein sequences, the BLAST Server (BLASTX, Altschul *et al.*, 1990) was used to convert the nucleotide sequence into the six possible protein sequences, and compare these with the sequences present in the data base.

The protein encoded by *mdbA* in reading frame +1, MdbA, was found to have significant sequence identity within residues 15-86 to amino acids (a.a.) 6-76 of the DNA binding H-NS protein from *Escherichia coli*, *Salmonella typhimurium*, *Serratia marcescens* (45-47% identity), *Proteus vulgaris* (a.a. 6-93, 39% identity), *Haemophilus influenzae*(a.a. 6-65, 37% identity), the *E. coli* StpA protein (a.a. 3-82, 45% identity) and the *Shigella flexneri* pathogenesis gene *kcpA* (a.a. 1-48, 47% identity) (Appendix 6).

The *mtfI* gene product was not found to have any similar sequences, however by manual comparisons a putative Fur box with high sequence similarity to consensus Fur binding sequences was identified in the -35 promoter region of *mtfI*, indicating that Mcc24 expression and/or Mcc24 immunity might be iron regulated (Figure 26).

Figure 24. Continued.

```
      σ70-10      RBS mftI
841   TAAGTTAACAAGAATGAGTTTTCTTAATTTTGCATTTTCTCCTGTATTCTTCTCCATTAT
                        METSerPheLeuAsnPheAlaPheSerProValPhePheSerIleMET

901   GGCGTGTTATTTCATTGTATGGAGAAATAAACGAAACGAATTTGTCTGCAATAGATTGCT
       AlaCysTyrPheIleValTrpArgAsnLysArgAsnGluPheValCysAsnArgLeuLeu

961   ATCAATTATAATAATATCTTTTTTGATATGCTTCATATATCCATGGCTAAATTACAAAAT
       SerIleIleIleIleSerPheLeuIleCysPheIleTyrProTrpLeuAsnTyrLysIle

1021  CGAAGTTAAATATTATATATTTGAACAGTTTTATCTTTTTTGTTTTTTATCGTCACTCGT
       GluValLysTyrTyrIlePheGluGlnPheTyrLeuPheCysPheLeuSerSerLeuVal

           σ70-35                  σS-10   σ70-10      RBS   mftS
1081  GGCTGTTGTAATAAACCTAATTGTATACTTTATATTATACAGGAGAATGTATATGAGAGAG
       AlaValValIleAsnLeuIleValTyrPheIleLeuTyrArgArgCysIle***
                                                    METTyrMETArgGlu

1141  TTAGATAGAGAGGAATTAAATTGCGTTGGTGGGGCTGGAGATCCGCTTGCAGATCCTAAT
      LeuAspArgGluGluLeuAsnCysValGlyGlyAlaGlyAspProLeuAlaAspProAsn
            Leader Peptide
                                                    ApaI
1201  TCCCAAATTGTAAGACAGATAATGTCTAATGCGGCATGGGGCCCGCCTTTGGTGCCAGAG
      SerGlnIleValArgGlnIleMETSerAsnAlaAlaTrpGlyProProLeuValProGlu

1261  CGGTTTAGGGGAATGGCTGTTGGAGCCGCAGGTGGGGTTACGCAGACAGTTCTTCAAGGA
      ArgPheArgGlyMETAlaValGlyAlaAlaGlyGlyValThrGlnThrValLeuGlnGly

           NdeI
1321  GCAGCAGCTCATATGCCGGTTAATGTCCCTATACCTAAAGTTCCGATGGGACCCTCATGG
      AlaAlaAlaHisMETProValAsnValProIleProLysValProMETGlyProSerTrp

1381  AACGGAAGTAAAGGATAAGATTCATTAAGGTAGAATATTTAACTTTGCTTGGGTGTTATA
      AsnGlySerLysGly***

1441  TCACTCAGGTAAAGTTATTACTCTATTCATCAGATAATTGGCTAATCTTTTTTAACAAGT

1501  AATTCTCTATGGTAATCGTGTACAGACACTATAGCTCTCATGGATTTGCTCCCTCTCCTC

1561  TGATTCTCCCCAGACTGTATTTGTCGTGTAAGTTCGGATAAGGAGAATAGGATCACCTGG

1621  CGCACTATTTTGAACTCCATCAGTAGCCACATGACCGTTGAAAGAGCCAAAGGGAAGCAA

1681  CTAAAGCTGTCTGATAAACAAAAAATTTGTAATGGATGCACGAAACCGGGCTGCACTGGT

1741  TGAATTATTTCCAGTCTAACGACCAATTGTCTATAGAACACCGATTCATAATAATATAAT
```

Figure 24. Continued.

σ^S-35/σ^{70}-35

1801 TATTTTTTACCATATCATCAGTCAGTAAGGAAAGAAAAGGTGTTTCGCCAAGAAGCAACA

σ^S-10 RBS *mftA* *EcoRI*

1861 GTATACAGGAAAATGAAGTGGTGCGGGCGGGCAGTATTATTGCCGGGAATTCCCCCCTGG

METLysTrpCysGlyArgAlaValLeuLeuProGlyIleProProTrp

1921 TTGGTCATATCAGTAAGCGTTTTTTTCTTTATTTCTTTTATAGTTTTTGTTATGGAAGGT

LeuValIleSerValSerValPhePhePheIleSerPheIleValPheValMETGluGly

1981 ACATACACACGCCGCATTAATGTAGAAGGAGAAATCACTACATGGCCCCGACCTGTAAAT

ThrTyrThrArgArgIleAsnValGluGlyGluIleThrThrTrpProArgProValAsn

2041 GTCTATTCAGGTGTACAGGGAGTTGTCATAAAACAGTTCGTCACTGAAGGACAAAGAATC

ValTyrSerGlyValGlnGlyValValIleLysGlnPheValThrGluGlyGlnArgIle

2101 AAAAAAGGCGACCCAATTTATCTGATTGATGTCAGTAAAAGTACTAGTAGTGGTGTTGTA

LysLysGlyAspProIleTyrLeuIleAspValSerLysSerThrSerSerGlyValVal

EcoRV *EcoRV*

2161 GGTGATAACAAGCGGCGAGATATCGAAAAGCAACTGTCCCGCATTGGGGATATCATATCC

GlyAspAsnLysArgArgAspIleGluLysGlnLeuSerArgIleGlyAspIleIleSer

2221 CGCCTTGAAGAAAATAAAAAAACTACACTACAGACACTGGAAAAACAACGGATTCAGTAC

ArgLeuGluGluAsnLysLysThrThrLeuGlnThrLeuGluLysGlnArgIleGlnTyr

2281 TACTCAGCTTTTGAACGTTCGACTGAAATCCTTCGTCGTGCTGAAGAAGGAGTAAAAATA

TyrSerAlaPheGluArgSerThrGluIleLeuArgArgAlaGluGluGlyValLysIle

2341 ATGAAAAGTAATATGGATAATTATAAACAATATCAAACGAAAGGACTCATAAACAAAGAC

METLysSerAsnMETAspAsnTyrLysGlnTyrGlnThrLysGlyLeuIleAsnLysAsp

2401 CAGTTAACCAACCAGATAGCGTTGTATTATCAGCAGCAAAATAACATATTGAGTCTGAGC

GlnLeuThrAsnGlnIleAlaLeuTyrTyrGlnGlnGlnAsnAsnIleLeuSerLeuSer

BstEII

2461 ACACAAAATGAACAAAACTTATTACAGGTAACCAGTCTGGAAAGCCAGATGCAGACCCTA

ThrGlnAsnGluGlnAsnLeuLeuGlnValThrSerLeuGluSerGlnMETGlnThrLeu

PstI

2521 GCAGCGGAATTTGACAACCGTATTTATCAGGTGGAACTGCAGCGTTACGAATTACAGAAA

AlaAlaGluPheAspAsnArgIleTyrGlnValGluLeuGlnArgTyrGluLeuGlnLys

2581 GAGCTGGTTGATACAGATGCTGGAAGAGATATTATTATTCGCGCTTTGTCAGATGGGAAA

GluLeuValAspThrAspAlaGlyArgAspIleIleIleArgAlaLeuSerAspGlyLys

Figure 24. Continued.

```
2641  ATTGATTCGTTGAGTGTTACACCGGGGCAGATGGTCAGCGTAGGCGACAGTCTGTTACAA
      IleAspSerLeuSerValThrProGlyGlnMETValSerValGlyAspSerLeuLeuGln

                                                        KpnI
2701  ATTATTCCAGAAGAAATAAAAAATTACCATCTGATTGTTTGGGTACCCAATAATGCCATA
      IleIleProGluGluIleLysAsnTyrHisLeuIleValTrpValProAsnAsnAlaIle

2761  CCTTATATTTCTGTTGGTGATAACGTTAATGTTCGTTATGAGGCCTTTCCGCCAGGAAAA
      ProTyrIleSerValGlyAspAsnValAsnValArgTyrGluAlaPheProProGlyLys

2821  TTTGGCCAGTTTACAGCAAAAATAATGTTAATATCCAGAACTCCGGCCTCCGCTCAAGAA
      PheGlyGlnPheThrAlaLysIleMETLeuIleSerArgThrProAlaSerAlaGlnGlu

2881  ATGCAGACCTATCCCGGAGCCCCAAGAAATAATACAGGTGTTTCTGTGCCTTACTACAAA
      METGlnThrTyrProGlyAlaProArgAsnAsnThrGlyValSerValProTyrTyrLys

2941  ATAGTGCTAAATCCGGAGCAACAAACAATAGAATACGGCGAAAAAAAAAATGCCTCTGGAG
      IleValLeuAsnProGluGlnGlnThrIleGluTyrGlyGluLysLysMETProLeuGlu

3001  AATGGTATGAAAGCACAGAGTACTTTATTTCTGGAAAAGAGGAAAATATACCAGTGGATG
      AsnGlyMETLysAlaGlnSerThrLeuPheLeuGluLysArgLysIleTyrGlnTrpMET

                                                  RBS  mftB
3061  CTATCCCCATTCTATAACATGAAATACAGTGCGGTGGGGCCAGTTAATGAACAATAACGC
      LeuSerProPheTyrAsnMETLysTyrSerAlaValGlyProValAsnGluGln***

                                                        METAsnAsnAsnAla

3121  TACCTCCCCCTTAAATACTCTCTTGAACAAACTAGAAATAGGACTACGGCGTCGCATTCC
       ThrSerProLeuAsnThrLeuLeuAsnLysLeuGluIleGlyLeuArgArgArgIlePro

3181  GGTCGTGCACCAGACTGAATCATCAGAATGTGGGCTGGCCTGTCTGTCGATGATATGTGG
       ValValHisGlnThrGluSerSerGluCysGlyLeuAlaCysLeuSerMETIleCysGly

         BglI
3241  CCACTATGGCAGGCATATTGATCTGAGTACTCTGCGTCGCCAGTTTAACCTGTCTGCTCT
       HisTyrGlyArgHisIleAspLeuSerThrLeuArgArgGlnPheAsnLeuSerAlaLeu

                                                        AvaI
3301  GGGAACAACTCTGGCTGGCATTACAGAAATAGGATCTCAATTGGGTATGGAAACCCGAGC
       GlyThrThrLeuAlaGlyIleThrGluIleGlySerGlnLeuGlyMETGluThrArgAla

3361  ATTCTCACTGGATCTAAATGAACTCAGTGTTCTGAAGTTGCCCTGCATCCTTCACTGGGA
       PheSerLeuAspLeuAsnGluLeuSerValLeuLysLeuProCysIleLeuHisTrpGlu
```

Figure 24. Continued.

```
3421  GTTTAGCCATTTTGTGGTGCTGGTCAGCGTCAGAAAAAATCATTTCGTTCTCCATGATCC
       PheSerHisPheValValLeuValSerValArgLysAsnHisPheValLeuHisAspPro

3481  GGCACGAGGACGTAGAACGGTAGGACTTGCTGAGATGTCACAGTGTTTTACCGGTGTCGC
       AlaArgGlyArgArgThrValGlyLeuAlaGluMETSerGlnCysPheThrGlyValAla

3541  GCTTGAAGTCTGGCCCGGCACAGAGTTTGTTCAGGAAACCATGAAGAACCGGGTAGTGCT
       LeuGluValTrpProGlyThrGluPheValGlnGluThrMETLysAsnArgValValLeu

          BstBI
3601  TCGAACGCTGTTTCGCAGCATTTATGGTCTCCGGAGTACGTTGACTAAAATTTTTTGCTT
       ArgThrLeuPheArgSerIleTyrGlyLeuArgSerThrLeuThrLysIlePheCysPhe

3661  TTCACTGGTGATTGAAGCTGTAGGTCTGGTGATACCTGTTGGCACTCAACTGGTGATGGA
       SerLeuValIleGluAlaValGlyLeuValIleProValGlyThrGlnLeuValMETAsp

                 PstI
3721  TCACGCCATTCCTGCAGGAGATCGAGGCCTGCTCTCCCTCATCTGCGTCGGTCTGATGTT
       HisAlaIleProAlaGlyAspArgGlyLeuLeuSerLeuIleCysValGlyLeuMETPhe

3781  TTTCATCCTGTTGCGCACCGCGGTCAGTATGATTCGCTCATGGTCGTCTTTGGTAATGGA
       PheIleLeuLeuArgThrAlaValSerMETIleArgSerTrpSerSerLeuValMETGlu

                                                      PstI
3841  AACGCTAATAAACGTGCAGTGGCAGTCGGGGTTGCACCGGCATCTGCTGCAGTTGCCGCT
       ThrLeuIleAsnValGlnTrpGlnSerGlyLeuHisArgHisLeuLeuGlnLeuProLeu

3901  GGCGTATTTTGAACGGCGAAAGATGGGTGATATTCAGTCCCGCTTCAGTTCTCTTGACAC
       AlaTyrPheGluArgArgLysMETGlyAspIleGlnSerArgPheSerSerLeuAspThr

3961  ACTGCGTACCACGTTTACAACCAGCGTGGTCGGAGCGATTATGGACAGCATCATGGTTTC
       LeuArgThrThrPheThrThrSerValValGlyAlaIleMETAspSerIleMETValSer

4021  CGGAGTCCTGGCTATGTTGGTGTTGTACGGTGGGTGGTTGACAACTATTGTGCTGGGTTT
       GlyValLeuAlaMETLeuValLeuTyrGlyGlyTrpLeuThrThrIleValLeuGlyPhe

4081  CACCATTATATATGTGCTAATTCGTTTACTGACCTATAACTATTATCGCCAGTTGTCGGA
       ThrIleIleTyrValLeuIleArgLeuLeuThrTyrAsnTyrTyrArgGlnLeuSerGlu

4141  AGAATCCCTGATAAGGGAAGCACGTGCCAGTTCGTATTTTATGGAAACCCTGTACGGAAT
       GluSerLeuIleArgGluAlaArgAlaSerSerTyrPheMETGluThrLeuTyrGlyIle

4201  AGCCACAATCAAGATGCAGGGAATGGGGGAGCGCAGAGGTCGTCACTGGCTGAATCTGAA
       AlaThrIleLysMETGlnGlyMETGlyGluArgArgGlyArgHisTrpLeuAsnLeuLys
```

Figure 24. Continued.

```
4261  GATTGATGCAATCAATACTGGTATAAGGCTTGCCAGGATGGATATGCTTTTCAGTGGGAT
        IleAspAlaIleAsnThrGlyIleArgLeuAlaArgMETAspMETLeuPheSerGlyIle

4321  TAACACGTTTGTTGCAGCATGTGACCAGGTCGTGATTCTGTGGCTGGGAACTAGCCTGGT
        AsnThrPheValAlaAlaCysAspGlnValValIleLeuTrpLeuGlyThrSerLeuVal

4381  GATTGACAATCAGATGACCATCGGAATGTTTGTGGCGTTCGGGGTATTCCGCGGGCAATT
        IleAspAsnGlnMETThrIleGlyMETPheValAlaPheGlyValPheArgGlyGlnPhe

4441  CTCGGACCGTGTTGGTTCCCTGACAAATTTTCTGCTTCAGTTGCGTATGATGAGTCTTCA
        SerAspArgValGlySerLeuThrAsnPheLeuLeuGlnLeuArgMETMETSerLeuHis

4501  TAATGAACGTATTGCTGATATAGCCATGAACGAGCGGGAAGCCCGGAAGCCTGATACGGC
        AsnGluArgIleAlaAspIleAlaMETAsnGluArgGluAlaArgLysProAspThrAla

4561  CATGAAAGCCGATATGTATCCGGTAGCACTGGAGACCCAGGACTTGAGCTTCCGTTATGA
        METLysAlaAspMETTyrProValAlaLeuGluThrGlnAspLeuSerPheArgTyrAsp

4621  CAGTCAGTCTGCTCCCGTTTTCAGCAACCTGAATATCAGTATAAAACCGGGAGAAAGTGT
        SerGlnSerAlaProValPheSerAsnLeuAsnIleSerIleLysProGlyGluSerVal

4681  GGCAATTACCGGGGCCTCAGGTTCAGGGAAAACCACCCTGATGAAGGTACTGTGTGGGCT
        AlaIleThrGlyAlaSerGlySerGlyLysThrThrLeuMETLysValLeuCysGlyLeu
                                  A site

                                                  KpnI  EcoRV
4741  GCTTGTACCCGAATCCGGCAGAGTCATGATAGACGGTACCGATATCAGATCGTTGGGGGT
        LeuValProGluSerGlyArgValMETIleAspGlyThrAspIleArgSerLeuGlyVal

4801  CAATAATTATCACAAAATTATTTCCTGTGTGATGCAAGATGACCGACTGTTTTCAGGCTC
        AsnAsnTyrHisLysIleIleSerCysValMETGlnAspAspArgLeuPheSerGlySer

4861  GATTAGAGAAAATATTTGCGGTTTCACTGAAAATATTGATGAGGCATGGATGGTGGAATG
        IleArgGluAsnIleCysGlyPheThrGluAsnIleAspGluAlaTrpMETValGluCys

              NheI
4921  TGCCAGAGCTAGCTTTATTCACGACGTTATCATAAAAATGCCTATGGGTTATGATACACT
        AlaArgAlaSerPheIleHisAspValIleIleLysMETProMETGlyTyrAspThrLeu

                                                          AvaI/XhoI
4981  GATAGGTGAACTGGGTGAAGGTCTTTCTGGCGGGCAGAAACAGCGAATTTTTATTGCTCG
        IleGlyGluLeuGlyGluGlyLeuSerGlyGlyGlnLysGlnArgIlePheIleAlaArg
                                  Linker peptide

                    SalI
5041  AGCGCTTTACCGTCGACCGGGCATTTTATTCATGGATGAGGCCACAAGTGCGCTGGATAC
        AlaLeuTyrArgArgProGlyIleLeuPheMETAspGluAlaThrSerAlaLeuAspThr
                                  B Site
```

Figure 24. Continued.

```
5101  TGAAAGTGAATATTATGTTAATCAGGCGATAAAGCAACTGAATATAACACGAATAATCAT
       GluSerGluTyrTyrValAsnGlnAlaIleLysGlnLeuAsnIleThrArgIleIleIle

5161  CGCACACAGAGAAACAACAGTAAAATCAGCCGACAGGATAATTTTACTGGAGGCGCCTGC
       AlaHisArgGluThrThrValLysSerAlaAspArgIleIleLeuLeuGluAlaProAla

                                                          Sau3AI
5221  GAGGATATAACTAAAATAATGATATTTATTACTCAAGGGTAGAGATC
       ArgIle***
```

Figure 24. The complete nucleotide sequence of the microcin 24 encoding region of pGOB18. All restriction enzymes with less than 5 sites are shown, except *Sau*3AI (13 sites) where only the first and last sites are shown indicating the start and end of the nucleotide sequence. The putative start sites of each gene product are shown in bold. The putative Fur binding site (Litwin and Calderwood, 1993), leader peptide (Håvarstein *et al.*, 1994), ATP binding sites (Fath and Kolter, 1993) and linker peptide (Koronakis *et al.*, 1995) are also shown in bold. The promoter sites indicated await experimental evaluation: Ribosome binding sites, RBS; putative σ^S dependent promoters, σ^S-10 and σ^S-35; putative σ^{70} dependent promoters, σ^{70}-10 and σ^{70}-35. Promoters have been named: P_{Mdb}, promoter for *mdbA*; P_1, promoter for *mtfI/mtfS*; P_2, promoter for *mtfS*; P_{AB}, promoter for *mtfAB*. The numbers on the left margins refer to nucleotide position. Genbank accession number U47048.

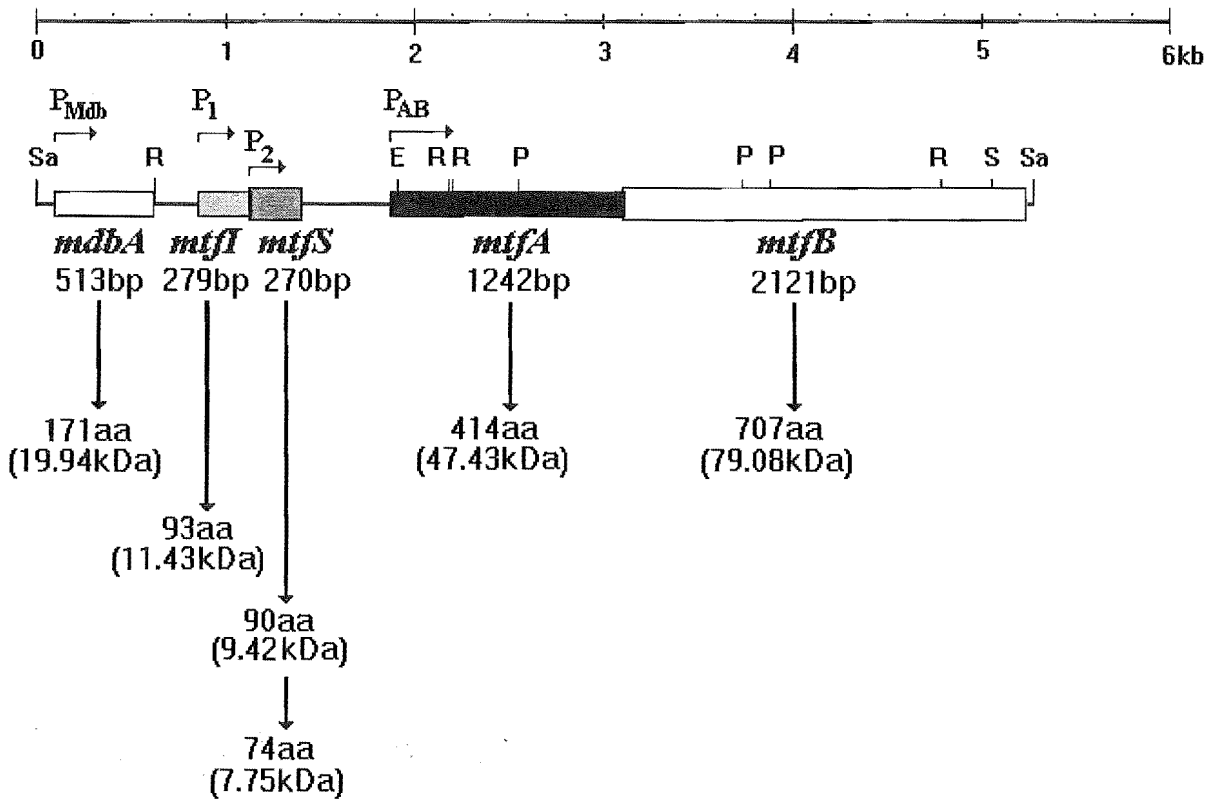


Figure 25. Physical map of the microcin 24 encoding region of pGOB18. The genes encoded by the 5267bp insert of pGOB18 are shown by boxes. Arrows indicate the direction of transcription with *mdbA*, *mtfI/mtfS* and *mtfA/mtfB* forming operons. The predicted number of amino acids and molecular weight in kilodaltons (kDa) is shown for each gene product. Promoter sequences were identified by DNA sequencing. The promoter P_1 is thought to control the transcription of both *mtfI* and *mtfS*. Restriction enzymes: E, *Eco*RI; P, *Pst*I; R, *Eco*RV; S, *Sal*I, Sa, *Sau*3AI.

P_1	792-	GATAATGATTTGCATTATT -810
Fur	5'-	GATAATGATAATCATTATC - 3'
Consensus		

Figure 26. Alignment of the P_1 promoter Fur-box with the Fur consensus sequence. Fur consensus sequence had previously been determined (Litwin and Calderwood, 1993). Lines indicate regions of symmetry. Numbers indicate nucleotide position of the Fur box in the DNA sequence (Figure 24).

Sequence comparisons to the *mtfS* gene product failed to indicate similarity to known bacteriocins, however manual comparisons to ColV identified a N-terminal double glycine leader sequence, a common feature of many small proteinaceous toxins (Figure 27), suggesting that MtfS might be subject to post-translational modification.

M Y M R E L D R E E L N C V G G	A G D P... MtfS
M R T L T L N E L D S V S G G	A S G R... CvaC
L S x x E L x x I x G G	Consensus
Δ x x Δ Σ x Σ Σ Δ x x Δ	sequence

Δ= conserved hydrophobic amino acids

Σ= conserved hydrophilic amino acids

Figure 27. Alignment of double glycine leader peptide sequences. The leader peptide of CvaC and the consensus sequence had been previously determined (Håvarstein *et al.*, 1994).

The gene product of *mtfA*, MtfA,was found to have a high degree of sequence similarity to the ColV transporter CvaA (71% identity, Appendix 6). Analysis of the *mtfB* gene product, MtfB, identified high sequence similarity (73% identity, Appendix 6) to the ColV transporter CvaB and other ATP-binding transport proteins. Analysis of the ATP-binding domain in MtfB showed that it was very similar to those in CvaB and the ATP-binding consensus sequence (Figure 28).

A Site	B Site	
GASGSGKTTLMKV	ILFMD	MtfB (microcin 24)
GASGAGKTTLMKV	ILFMD	CvaB (colicin V)
GRSGSGKST	IL I LD	Consensus sequence

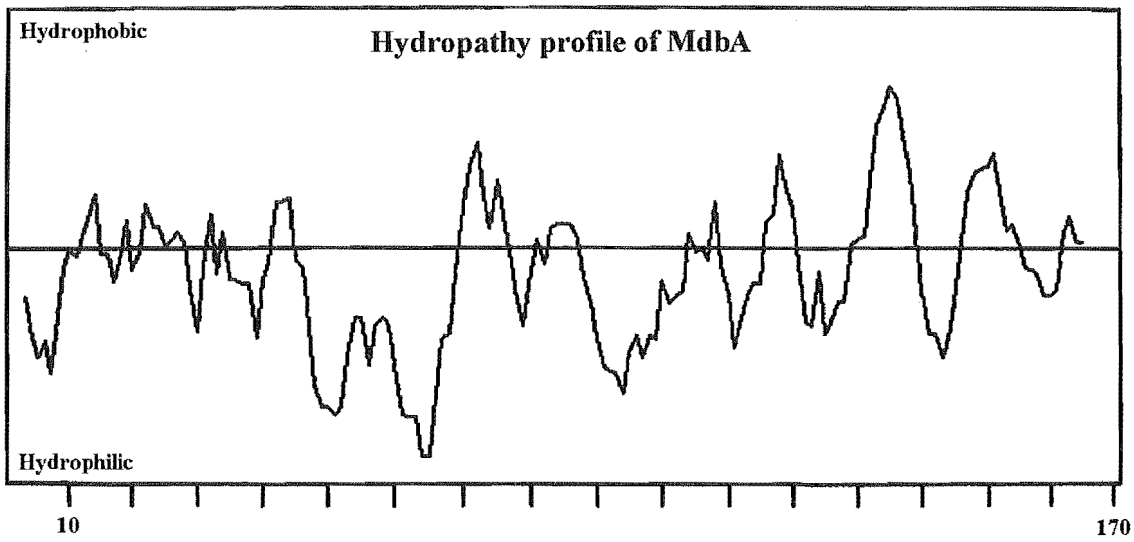
Figure 28. Alignment of ATP-binding domains for MtfB, CvaB and the ATP-binding consensus sequence. ATP binding domains for CvaB and the consensus sequence had been previously determined by Gilson *et al.*, (1990) and Fath and Kolter, (1993) respectively.

3.2.4 HYDROPATHIC PROFILES OF pGOB18 OPEN READING FRAMES.

The hydrophobic force is the force arising from the strong cohesion of a solvent, which drives molecules lacking any favourable interactions with water molecules themselves from the aqueous phase. For proteins, the native structure will therefore remove the greatest number of hydrophobic side chains from exposure to water at the same time as removing the least number of hydrophilic residues. Kyte and Doolittle (1982) derived a hydropathy scale in which each amino acid was assigned a value reflecting its relative hydrophobicity and hydrophilicity, by considering the hydrophobic and hydrophilic properties of the 20 amino acid side chains. A computer program utilising a moving segment of predetermined length and determining the average hydropathy of the segment from the amino terminus to the carboxy terminus was devised. For globular proteins it was found that interior portions were represented by regions on the hydrophobic side and exterior portions by regions on the hydrophilic side. It was also found that for membrane bound proteins, large uninterrupted regions on the hydrophobic side of the midpoint line represented portions located in the lipid bilayer. As such, this technique has proved very useful in determining the number and location of membrane spanning domains in open reading frames identified by DNA sequencing.

In order to understand the physical characteristics of the proteins encoded by the nucleotide sequence, the open reading frames were each subjected to hydropathy analysis using TGREASE (FASTA; Pearson and Lipman, 1988) and the profiles are presented in Figure 29. The most striking feature of MdbA is a hydrophilic domain between residues 40-70. MtfI appears to consists of a N-terminal hydrophilic domain followed by two large hydrophobic domains which are likely to be inserted into the cytoplasmic membrane. The pre-MtfS peptide appears to be slightly hydrophobic and has a N-terminal leader peptide which is hydrophilic. Both transport proteins give the expected profile for proteins involved in ABC mediated transport. MtfA has the characteristic hydrophobic N-terminus which may anchor the protein to the inner membrane, a hydrophilic central region which could span the periplasmic space and a hydrophobic C-terminus which may form the outer membrane domain. The ABC transporter, MtfB, has multiple hydrophobic membrane spanning domains.

A.



B.

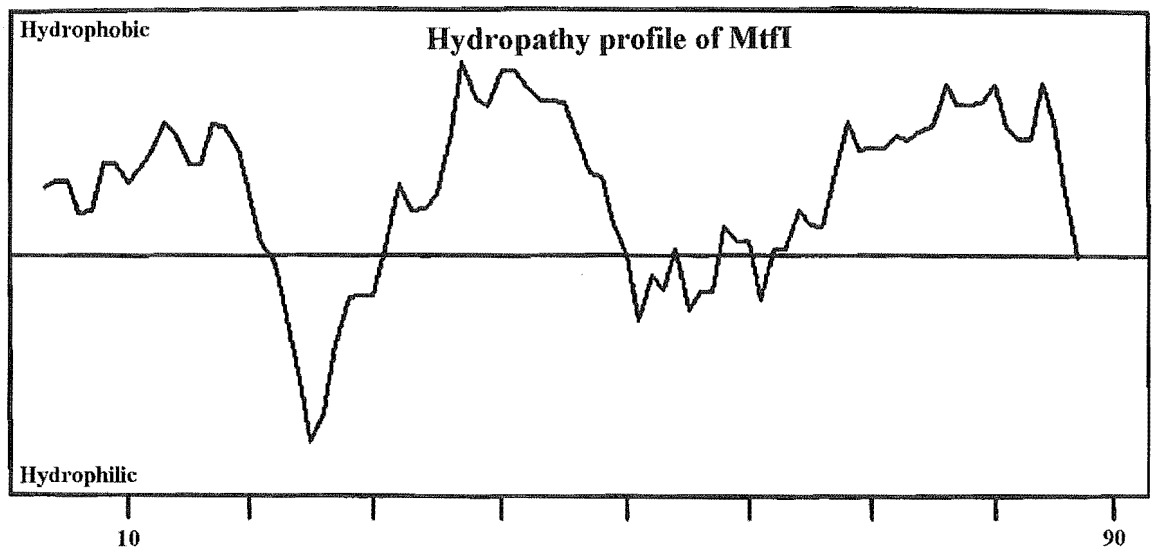
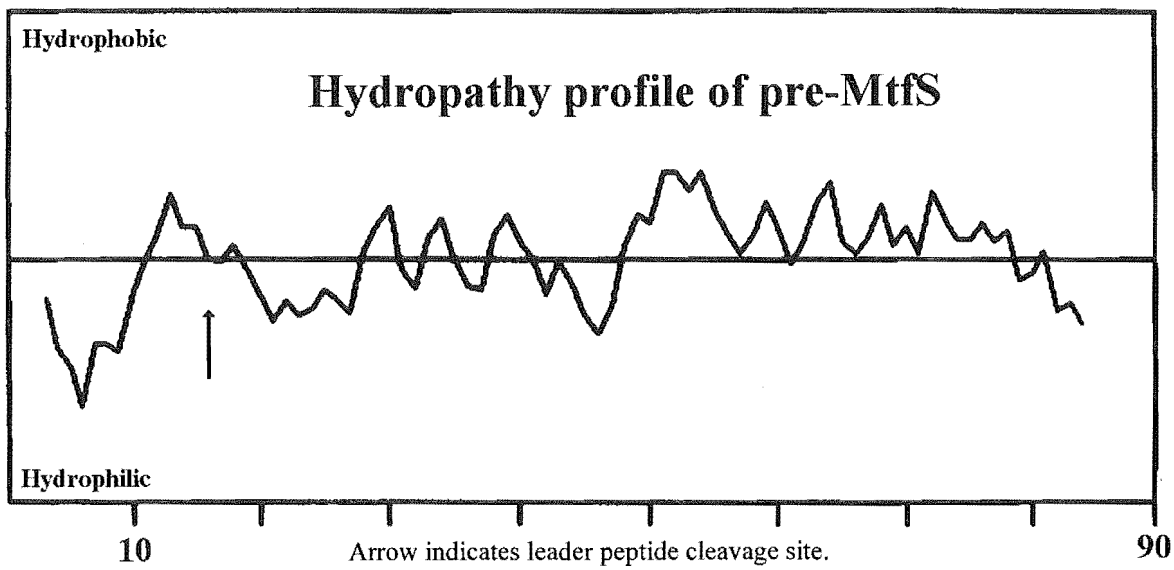


Figure 29. Continued.

C.



D.

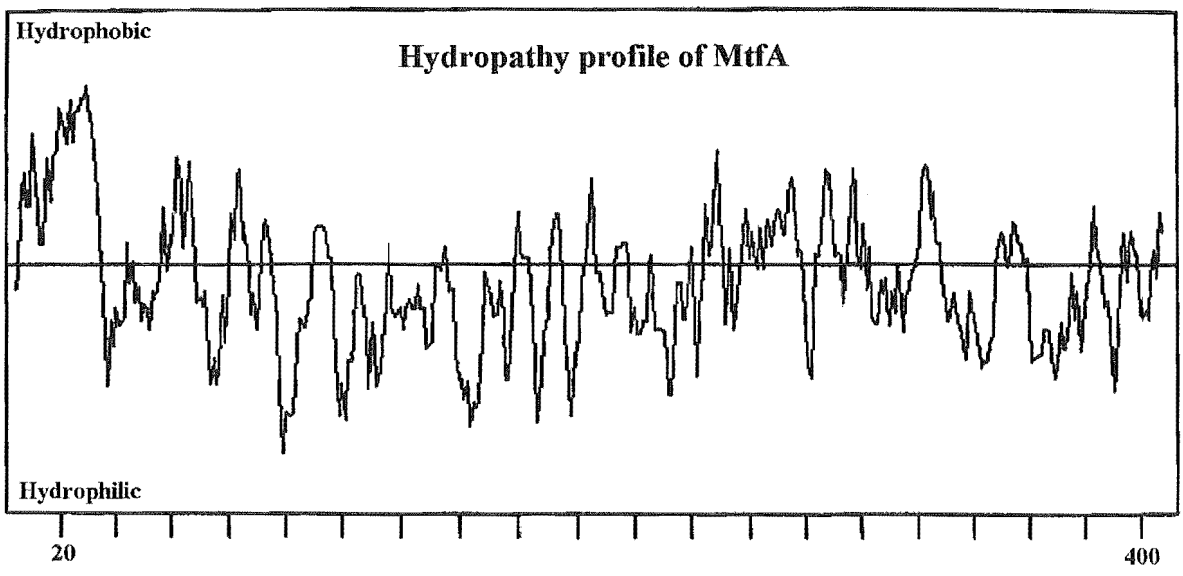


Figure 29. Continued.

E.

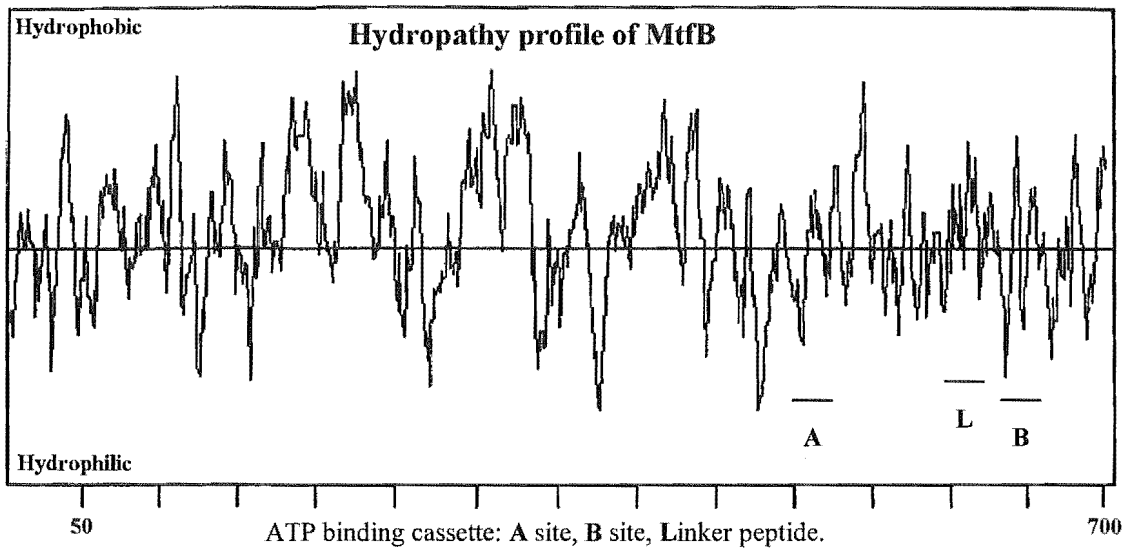


Figure 29. Hydropathy profiles of pGOB18 open reading frames. The number of amino acids for each protein is indicated on the x-axis.

3.3 FUNCTIONAL ANALYSIS OF *mtfS*, *mtfI* AND *mdbA*.

3.3.1 DETERMINATION OF GENE FUNCTION.

On the basis of trans-complementation with *cvaAB* (Results 3.1.3) *mtfA* and *mtfB* had been identified as the Mcc24 transport genes, however the functional characterisation of the structural and immunity genes had not been determined. DNA sequencing identified three open reading frames *mtf*I, *mtf*S and *mdb*A (Figure 25) that were located within the region previously determined by complementation to encode the Mcc24 structural and immunity genes (Results 3.1.5).

In order to determine the function of these three open reading frames, the Tet^R/Suc^R and $Kan^R/Strep^R$ nested deletions obtained from pGΔ813 (Results 3.2.2) were tested for Mcc24 immunity using the streak test against MC4100(pGOB18) on a LB plate containing 2,2'-dipyridyl, an iron chelator which is known to induce microcin

synthesis. Confirmation of immunity was checked by using the derivative as a lawn in the patch test. Mcc 24 activity was tested by trans-complementation with pHK22-6 (*cvaAB*$^{+}$) and assaying for activity using the patch test. Deletions encoding *mtfI* and *mtfS* were found to produce and be immune to Mcc24. The derivative T14 failed to grow on the M63 test plates during complementation and was re-assayed on LB+2,2'-dipyridyl overlay plate. A small fuzzy halo was produced on the MC4100 lawn after overnight incubation, suggesting that mtfS encoded Mcc24 activity.

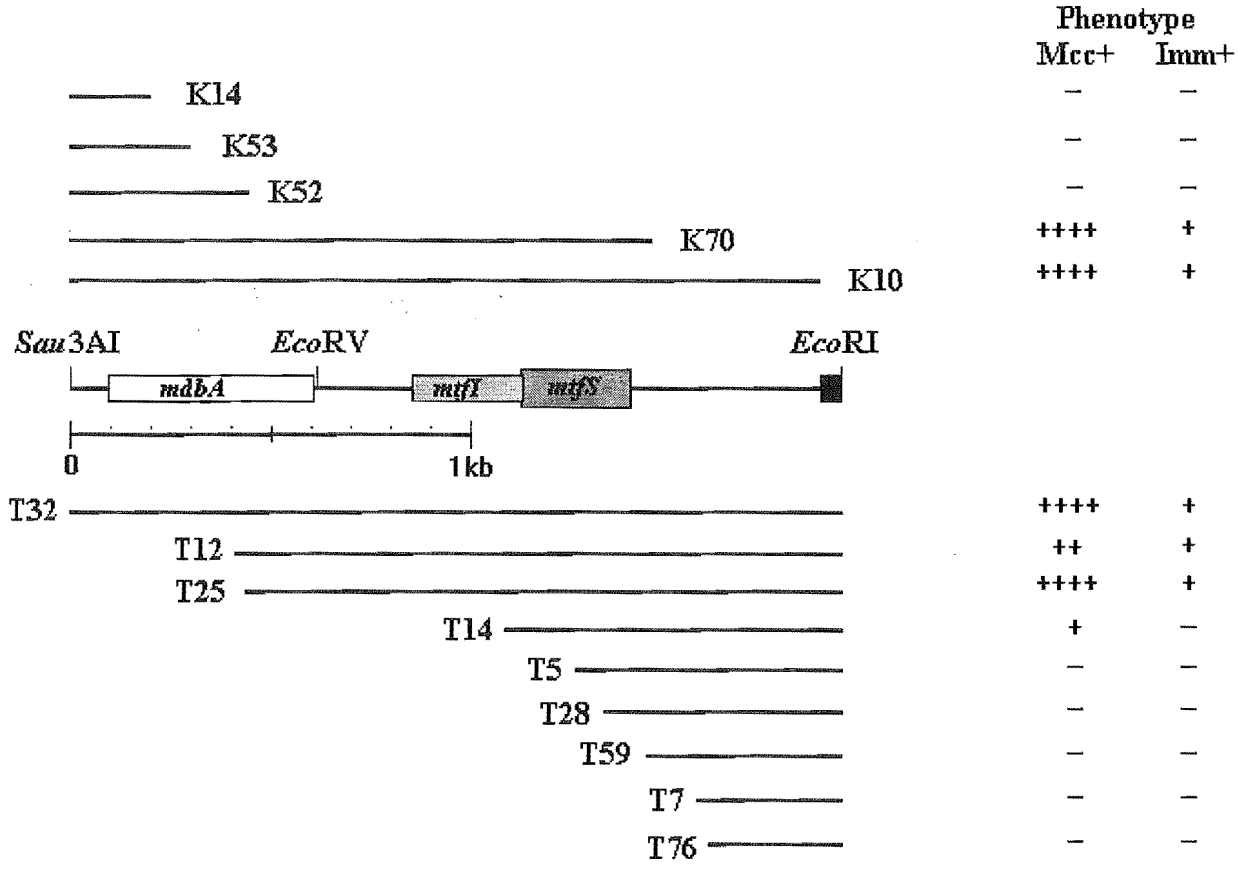


Figure 30. Trans-complementation between pGΔ813 nested deletions and colicin V transport proteins. Nested deletions from pGΔ813 were complemented with pHK22-6 and screened for microcin production using the patch test and microcin immunity using the streak test against MC4100(pGOB18). Mcc24 expression scale: + faint halo on LB+2,2'-dipyridyl (0.1mM); ++, 1.5mm halo; ++++, wild type expression (2.5mm halo). Immune and immunity deficient derivatives are denoted "+" and "-" respectively. Lines represent portion of nucleotide sequence retained after deletion.

*3.3.2 SATURATION MUTAGENESIS USING MINI-TN*10 TET.

Analysis of pGΔ813 deletions indicated that *mtfS* and *mtfI* encoded Mcc24 and Mcc24 specific immunity respectively. To confirm this and to elucidate the role of *mdbA* in Mcc 24 expression, mini-Tn*10 tet* insertions into *mtfS*, *mtfI* and *mdbA* were to be generated in compatible plasmid backgrounds and the resulting phenotype tested by trans-complementation.

A pBR322 based derivative, pGOB181, encoding this region had already been generated (Figure12B), however it was necessary to produce a compatible pACYC184 based derivative. The insert DNA form pGΔ813 (Appendix 4) was transferred to pACYC184 by the digesting of both with *Bam*HI and ligation. The resulting derivative, pLOB813 was found to be Mcc24 immune and produced Mcc24 when complemented with pHK11-1 (data not shown).

Mutagenesis of MC4100(pGOB181) and MC4100(pLOB813) was performed using λ1323 (Methods 2.5.1). In order to identify structural and immunity mutants, a 2μl aliquot of DNA from each of the pGOB181::mini-Tn*10* and pLOB813::mini-Tn*10* DNA pools was electroporated into MC4100(pHK22-6) or MC4100(pHK11-1) respectively. A total of 1000 MC4100(pHK22-6/pGOB181::mini-Tn*10*) transformants were tested for Mcc24 production using the patch test producing 63.1% $Mcc24^{+}$, 8.9% $Mcc24^{RED}$ and 28% $Mcc24^{-}$ insertions. Less MC4100(pHK11-1/pLOB813::mini-Tn10) transformants were obtained with 44.9%, 6.2% and 38.9% of the 272 transformants being $Mcc24^{-}$, $Mcc24^{RED}$ and $Mcc24^{+}$ respectively.

The insert positions of 50 $Mcc24^{-}$ and 30 $Mcc24^{RED}$ pGOB181::mini-Tn*10* mutants and 40 $Mcc24^{-}$ and 20 $Mcc24^{RED}$ pLOB813::mini-Tn*10* insertions were determined by restriction analysis and gel electrophoresis. A total of 15 independent $Mcc24^{-}$ and 5 $Mcc24^{RED}$ insertions were identified into pGOB181 and 12, $Mcc24^{-}$ and 3 $Mcc24^{RED}$ insertions identified into pLOB813 (Figure 31). DNA sequencing was used to located the exact position of pGOB181 insertions # 2, 12, 13, 14, 15 (all $Mcc24^{-}$) and pLOB813 insertions #1, 2, 11 (all $Mcc24^{-}$), 1r and 3r (both $Mcc24^{RED}$). The insertion points in the nucleotide sequence (Figure 24) were located after bases 1162, 1295, 1304, 1338, 1378, 248, 833, 1369, 794, and 1032 respectively. By re-testing the microcin activity and immunity of the independent mutations, it was found that all of the

pGOB181::mini-Tn*10* Mcc24^{RED} mutants were located within the *mtfI* gene or promoter producing a Mcc24 sensitive phenotype, whereas all of the Mcc24$^-$ mutants were inserted within *mtfS* but were still Mcc24 immune. The pLOB813::mini-Tn*10* insertions produced a similar pattern except the Mcc24$^-$ insertions #1 (Mcc24 immune) and #2 (Mcc24 sensitive) were located within *mdb*A and the *mtfI* promoter region respectively (Figure 31). The full phenotype of each mutant is shown in Table 6.

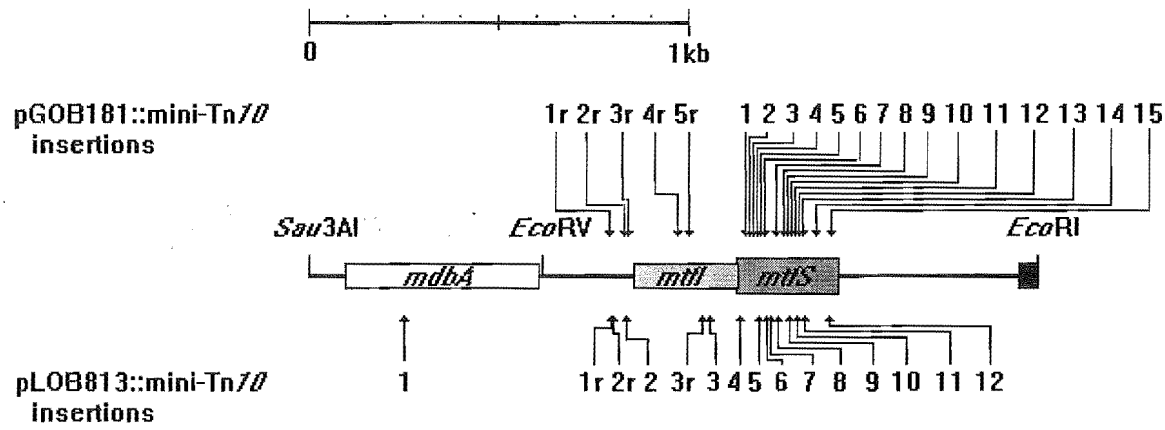


Figure 31. Location of mini-Tn*10* insertions in plasmids pGOB181 and pLOB813. Individual insertions (indicated by arrows) were mapped using *Eco*RI and *Eco*RI/*Hin*dII digestion followed by agarose gel electrophoresis. The insertion points of mutants 2, 12, 13, 14, 15 (pGOB181) and 1, 2, 11, 1r, 3r (pLOB813) were confirmed by sequencing.

Table 6. Phenotype of mini-Tn*10* insertions into pGOB181 and pLOB813.

	pGOB181::mini-Tn*10* insertions.				pLOB813::mini-Tn*10* insertions.		
	Mcc24 activity	Mcc24 immunity	Colony morphology		Mcc24 activity	Mcc24 immunity	Colony morphology
1	-	+	normal	1	-	+	normal
2	- a	+	normal	2	-	-	small
3	-	+ weak	normal	3	- a	+ w	normal
4	-	+	normal	4	-	+ w	normal
5	- a	+	normal	5	-	+	normal
6	- a	+	normal	6	-	+	normal
7	- a	+	normal	7	-	+ w	normal
8	-	+	small	8	-	+	normal
9	-	+	small	9	-	+	normal
10	- a	+	normal	10	1	+	normal
11	-	+	normal	11	- a	+	normal
12	- a	+	normal	12	-	+ w	translucent
13	- a	+	normal	1r	0.5mm	-	translucent
14	- a	+	normal	2r	0.5mm	-	normal
15	-	+	small	3r	0.5mm	+	small translucent
1r	0.5mm	-	normal				
2r	0.5mm	-	normal				
3r	1mm	-	normal				
4r	2mm	- b	normal				
5r	0.5mm	-	normal				

Positive phenotype denoted "+", negative phenotype denoted "-". Microcin activity tested using the patch test on M63 media and LB+ 2,2'-dipyridyl. Normal halo size pGOB181/pHK22-6, 2.5mm; pLOB813/pHK11-1, 2mm. Cell sizes were determined visually after overnight growth. Immunity was tested using cross streak test against MC4100(pGOB18).

[a] Translucent colony on LB + 2,2-dipyridyl plate. [b] MC4100(pGOB18) produced a small halo (1mm) when 4r was used as the bacterial lawn in the patch test compared to normal halo of 2.5mm on sensitive lawns. [w] Weak immunity defined as Mcc24 sensitive when tested using the streak test, but mcc24 resistant when mutant strain is used as the bacterial lawn in the patch test.

3.3.3 IDENTIFICATION OF mdbA, mtfI *AND* mtfS *GENE PRODUCTS.*

Analysis of the nucleotide sequence for *mdbA*, *mtfI* and *mtfS* predicted that these genes encoded proteins of 19.94, 11.43 and 9.42 kDa respectively (Figure 25), however the size of these proteins had not been confirmed by SDS-PAGE. The gene sequence for *mtfS* also indicated the presence of a double glycine leader sequence, implying that MtfS would be subject to post-translational modification. In order to confirm these predictions, the proteins encoded by wild type gene sequences were labelled with ^{35}S methionine/cysteine and compared to the proteins produced by various mini-Tn*10* insertions using SDS-PAGE maximised to visualise small proteins.

Protein analysis using maxicells.

The ability of *E. coli* strains carrying mutations in the major DNA repair genes *recA* and *uvrA* to synthesize plasmid encoded proteins was first described by Sancar *et al.*, 1979. It was found that irradiating the strain CSH603 (*recA1 uvrA6 phr-1*) stopped synthesis of chromosomal DNA but plasmid DNA that did not contain a UV hit was replicated. These non-dividing cells termed "maxicells" were then able to use plasmid DNA as the template for transcription resulting in differential labelling of plasmid encoded proteins with ^{35}S-labelled amino acids. Because of the difficulty in maintaining *recA uvrA* strains, *E.coli* strains carrying a mutation in *recA* alone have been used to generate maxicells (Pritchard and Holland, 1985). Although these strains are less sensitive to UV, by increasing the UV dosage and checking the efficiency of irradiation, it was possible to generate maxicells with the same efficiency as CSH603.

In order to analyse the proteins encoded by *mdbA*, *mtfI* and *mtfS*, maxicells were generated from *E. coli* MC4100 (*recA-56*) strains containing various Mcc24 plasmids. The required UV dosage for the generation of MC4100 maxicells was determined by irradiating both MC4100 and MC4100(pBR322) with increasing doses of UV (254nm) and determining viable cell count (Methods 2.8.2). It was found that MC4100(pBR322) was more UV sensitive than MC4100 alone (Figure 32) and a dosage of 200μJcm^{-2} (2Jm^{-2}) was chosen as the irradiation level for maxicell generation. Maxicells were prepared from MC4100 and MC4100 containing pBR322 and pACYC184 as controls as well as from MC4100 harbouring pGOB18, pGOB181 various and pGOB181::mini-

Tn*10* insertions, however after several attemps it was not possible to clearly define the proteins encoded by *mdbA, mtfS* and *mtfI* by SDS-PAGE.

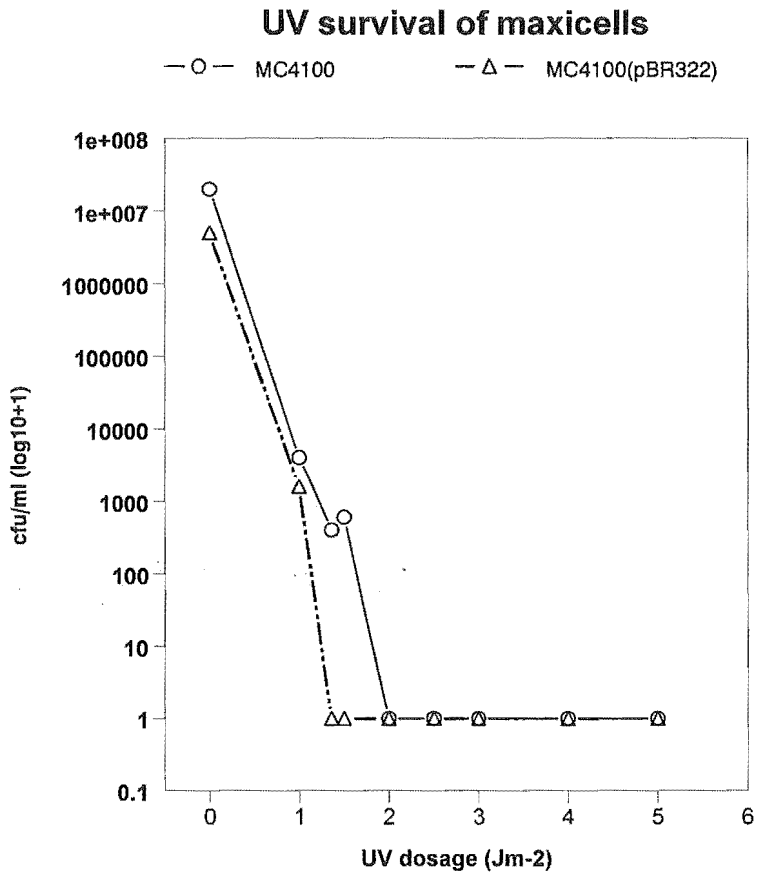


Figure 32. UV survival rate of MC4100. Exponential growing cultures (OD_{450} = 0.5) were irradiated with UV (254nm) at doses of 100, 136, 150, 200, 250, 300, 400, 500μJcm^{-2} and the viable cell number determined and plotted against UV dose.

Expression of *mdbA*, *mtfI* and *mtfS* using minicells.

Because protein expression using maxicells did not produce the desired result, the same plasmids that were used in the maxicell experiment were expressed in the minicell strain P678-54T. Minicells were prepared and labelled as previously described (Methods 2.8.1) and the proteins analysed in a 18% SDS-PAGE maximised for small proteins (Methods 2.7.3). Protein bands were identified with approximate molecular weights of 44kDa, 40kDa, 20kDa, 11.5kDa, 9.5kDa, and 7.5kDa (Figure 33).

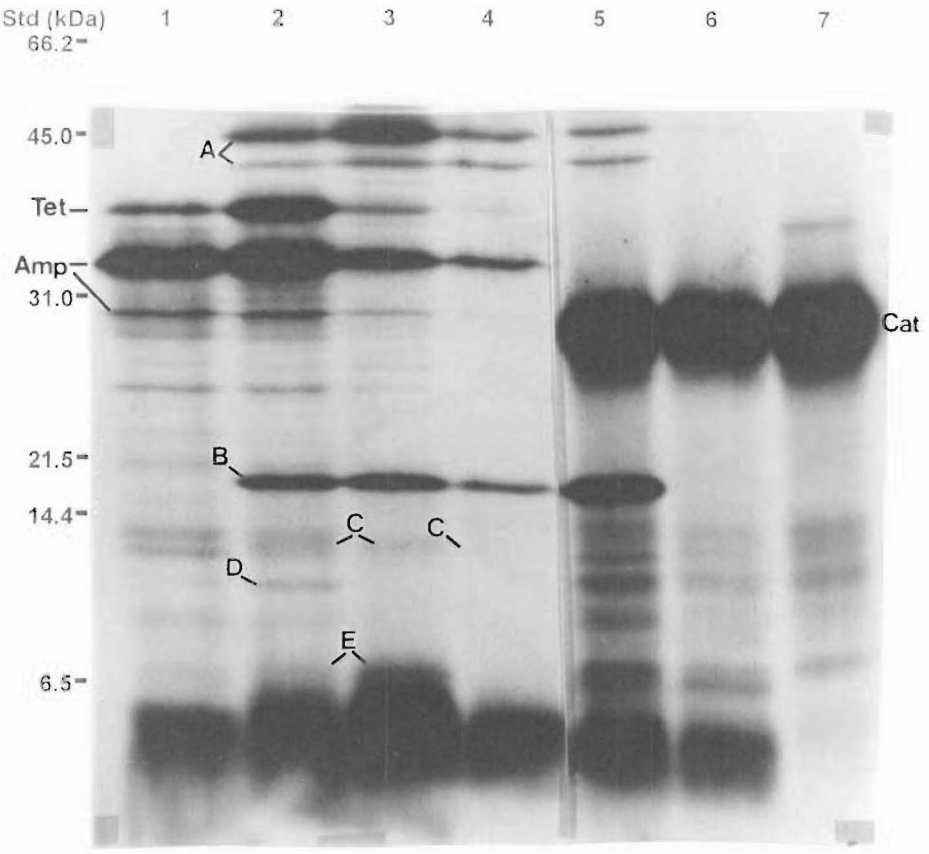


Figure 33. Expression of *mdb*A, *mtf*I and *mtf*S using minicells. The proteins were labelled with ^{35}S methionine/cysteine (Promix, Amersham) and analysised by SDS-PAGE in a 18% acrylamide gel. Lanes: 1, pBR322; 2, pGOB18; 3, pGOB181; 4, pGOB181::mini-Tn*10* #2 (*mtfS*⁻), 5, pLOB813::mini-Tn*10* #3r (*mtfI*⁻); 6, pLOB813::mini-Tn*10* #1 (*mdbA*⁻); 7, pACYC184. Protein bands indicated by arrows: A, MdbA dimer isomers 44kDa and 40kDa; B, MdbA monomer 20kDa; C, MtfI 11.5kDa; D, pre-MtfS 9.5kDa; E, MtfS 7.5kDa. Amp, β-lactamase 31kDa and 28kDa; Tet, TetR protein 37kDa (Sancar *et al.*, 1979). CAT, chloramphenicol. acetyltransferase 29.8kDa (Mayo *et al.*, 1988).

3.4 ANALYSIS OF MICROCIN 24.

3.4.1 PLATE ASSAYS FOR MICROCIN 24 ACTIVITY.

Prior to the analysis of the DNA sequence (Results 3.2.3), Mcc24 was classified as a colicin (O'Brien and Mahanty, 1994). In order to confirm the nature of this antimicrobial agent, plate assays using an MC4100 lawn (Methods 2.3.3) were repeated to determine if this antibiotic could pass through cellophane, the classical test for microcin activity (Arsensio *et al.*, 1976). The Mcc24 activity produced by MC4100(pGOB18) was found to dialyse through cellophane (Figure 34) confirming the classification of Mcc24.

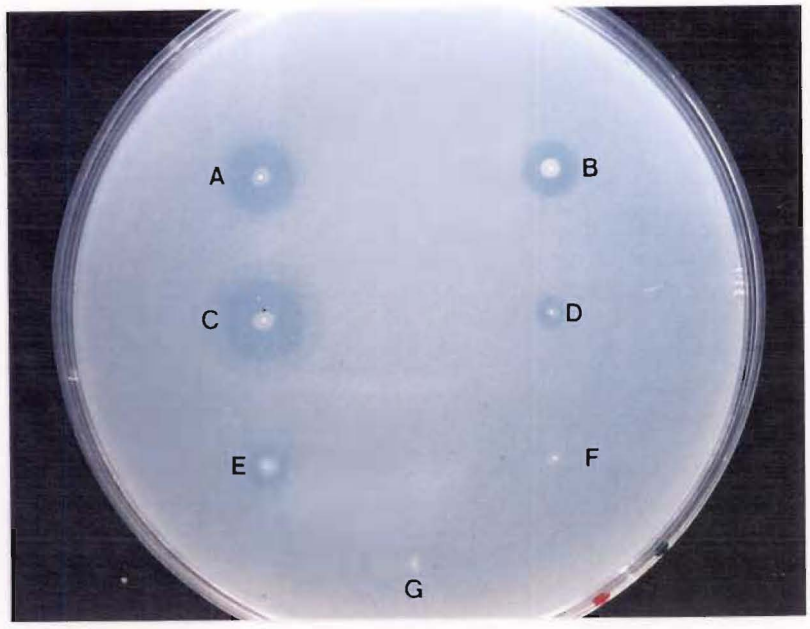
3.4.2 PREPARATION OF MICROCIN 24 EXTRACTS.

In order to analyse the antibiotic function of Mcc24, it was first necessary to extract sufficient protein from producing cells for use in further experiments, so several methods were attempted to determine which would give the best yield of the antibiotic. Mcc24 extracts were prepared from LB broth cultures induced with mitomycin C (Methods 2.9.1) and protein profiles determined in a 10 or 12.5% acrylamide gel (Methods 2.7.1) because during early trials it was still thought that Mcc24 was a colicin. The Mcc24 titre of extracts was determined by the critical dilution test (Methods 2.10.1).

Gel Filtration.

The first method attempted for purifying Mcc24 was gel filtration using a Sephadex G-100 column (Methods 2.9.6). The bed volume and V_o were determined to be 198cm^3 and 44.5ml respectively. After extraction the protein was precipitated using acetone and dissolved in 15ml 50mM KH_2PO_4, pH 7.0. A 2ml aliquot of Mcc24 extract (8 A.U./ml) was applied to the column and fractions concentrated and assayed for activity. Fractions 21-27 were found to inhibit growth of a MC4100 lawn and fraction 24 gave the strongest activity. SDS-PAGE analysis of the fractions in a 12.5% acrylamide gel with silver staining showed that fractions 21 and 22 contained 10 bands, fraction 23 contained 4 bands and fractions 24-27 contained 1 band of between 14 - 90 kDa. The total extract contained 16 bands.

A.



B.

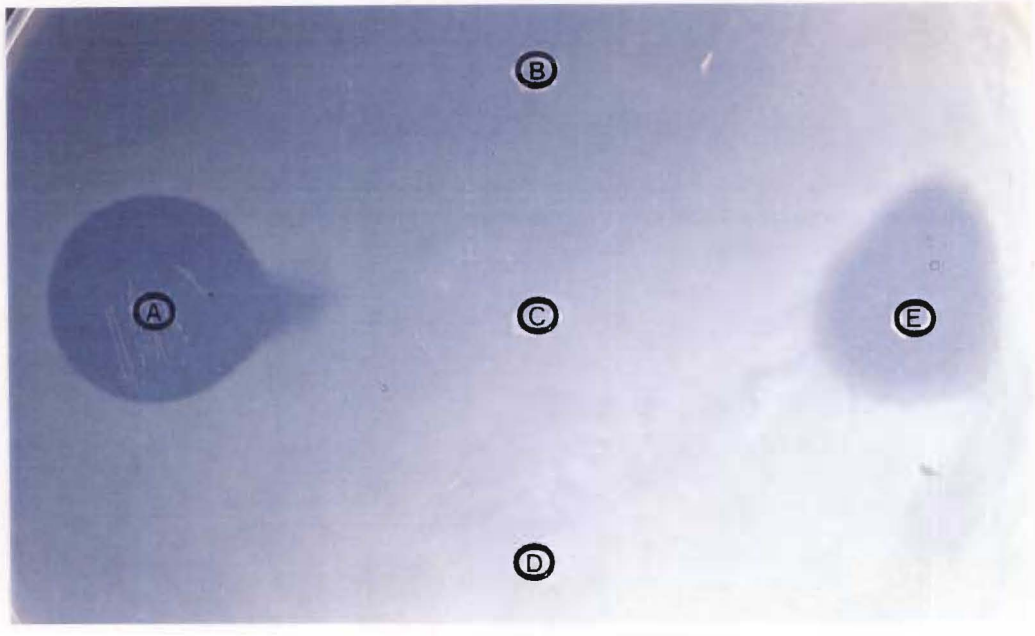


Figure 34. Plate assays for microcin 24 activity. A. Patch test. Strains: A, MC4100(pGOB18 Mcc24$^+$); B, MC4100(p24-2 Mcc24$^+$); C, MC4100(pHK11 ColV$^+$); D, MC4100(pColV-K30::Tn*10* ColV$^+$); E, BzB2101(pColA-CA31 ColA$^+$); F, MC4100(pBR322); G, MC4100. **B.** Cellophane test. A 0.2µl aliquot of overnight culture was placed onto a sterile cellophane filter on a M63 agar plate, incubated overnight and the cellophane removed and the plate overlayed. Zones of inhibition were identified after overnight incubation. Strains: A, MC4100(pHK11 ColV$^+$); B, MC4100; C, MC4100(pBR322); D, BzB2101(pColA-CA31 ColA$^+$); E, MC4100(pGOB18 Mcc24$^+$).

Ammonium Sulphate Precipitation.

Ammonium sulphate was added to varying saturations to induced culture supernatants (Methods 2.9.4) and the precipitate at each step dissolved in 200µl 50mM KH_2PO_4 (pH 7.0). A 10µl aliquot from each Mcc24 extraction was placed onto a lawn of MC4100 and inhibition of growth seen for all fractions. Microcin 24 extracts prepared using ammonium sulphate at 50% and 60% saturation gave the best inhibition. Activity dropped off sharply in fractions from saturations 60%. Analysis of the fractions by SDS-PAGE showed that the 25%, 40%, 50% and 60% saturation fractions contained 15 protein bands of molecular weights between 14 - 90 kDa.

Ion Exchange Chromatography.

Microcin 24-containing supernatants were dialysed against 50mM HEPES pH 7.6 (SP column) or 20mM Tris-HCl pH 8.2 (Q column). The 1ml ion exchange columns (Pharmacia) were prepared (Methods 2.9.7) and the samples applied to the columns. After the application, washing and elution steps the eluate was collected, precipitated (Methods 2.9.3) and the precipitate dissolved in 100µl of buffer. A 10µl aliquot was placed onto a lawn of MC4100 to assay for antimicrobial activity. Faint activity was seen in the SP column application fraction and very strong activity was seen in the Q column elution fraction. All of the fractions were analysed by SDS-PAGE in a 10% acrylamide gel. The elution fraction (Q column) was found to contain 4 proteins of molecular weights 45 kDa, 94 kDa, 98 kDa and 100kDa.

Extraction through Nitrocellulose Filters.

Extraction through nitrocellulose filters (Methods 2.9.5) was found to be an excellent method of Mcc24 extraction. After removal of the protein from the filters and dissolving the protein in buffer, a 10µl aliquot was found to retain antimicrobial activity. SDS-PAGE in a 10% acrylamide gel showed that this fraction contained 10 proteins of molecular weights between 14 - 90 kDa.

Once it was confirmed that Mcc24 was in fact a microcin, bulk extraction was performed by growing MC4100(pGOB18) in M63 medium for 6 hours (Methods 2.9.2). Dissolving the final precipitate in 1/1000th the original volume produced extracts with activity as high as 128 A.U./ml (Figure 35A). Bacterial growth in the clearing zones

during critical dilution tests occurred. To ensure that these colonies were not contaminants, the colonies were purified and tested for Mcc24 activity and immunity using the patch and cross streak tests. No zones of inhibition were produced by these colonies, but they were resistant to Mcc24 indicating they were spontaneous MC4100 mutants, resistant to Mcc24. SDS-PAGE maximised for small proteins showed that many proteins were present in the extract, including a band at the expected 7.75 kDa (Figure 35B). Testing for microcin activity after SDS-PAGE (Methods 2.10.2) failed to show any inhibition of the MC4100 lawn, unlike ColV which has been shown to retain activity in acrylamide gels (Fath *et al.*, 1994).

3.4.3 EFFECT OF PROTEINASE OR RNASE TREATMENT ON MCC 24.

To confirm that Mcc24 was a proteinaceous bacteriocin and did not contain a RNA component, activity of MC4100(pGOB18) was tested in the presence of proteinase K and RNase A (Methods 2.10.4). It was found that the presence of proteinase K inhibited MC4100(pGOB18) from killing a lawn of MC4100, however the presence of RNase A had no effect on Mcc24 activity.

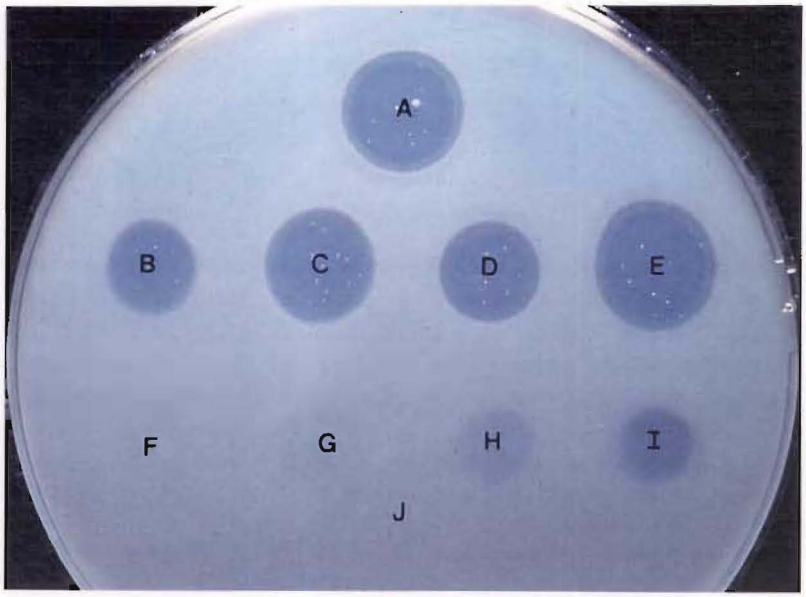
3.4.4 EFFECT OF CHLOROFORM ON MICROCIN 24 ACTIVITY.

Previously it had been shown that chloroforming transport mutants failed to restore Mcc24 activity (Results 3.1.2). To confirm that chloroform did not affect Mcc24, MC4100(pGOB18) was mixed with chloroform and the supernatant assayed for microcin activity (Methods 2.10.3). Chloroform was found to have no effect on the antimicrobial activity of Mcc24 present in the supernatant.

3.4.5 TIME SERIES

In order to determine the time required for Mcc24 to reduce the viability of a culture of a sensitive bacterium, a time series experiment was undertaken (Methods 2.10.5). Microcin extract was prepared and found to have an activity of 128 A.U./ml. After two hours incubation, it was found that the concentration of the sensitive cells was reduced by half (Figure 36).

A.



B.

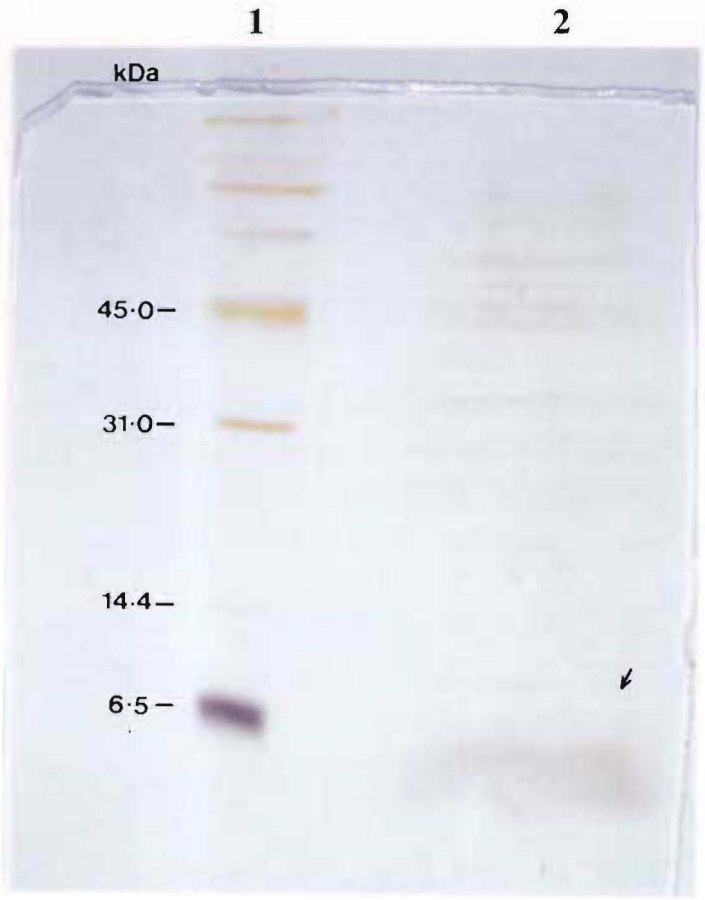


Figure 35. Extraction of microcin 24 using nitrocellulose filters. A. Estimation of microcin titre by critical dilution. Halos: A, Mcc24 extract undiluted; B-E, Mcc24 dilutions 1/16, 1/8, 1/4, 1/2 respectively; F-I, Mcc24 dilutions 1/256, 1/128, 1/64, 1/32 respectively; J, 0.1M Phosphate Buffer pH 7.4. **B.** SDS-PAGE of Mcc24 extract. The Mcc24 extract was analysed by SDS-PAGE in a 18% acrylamide gel maximised for small proteins and the protein bands were visualised by silver staining. Lanes: 1, BioRad Broad Range molecular weight standard; 2, Microcin 24 extract. Arrow indicates putative Mcc24 band.

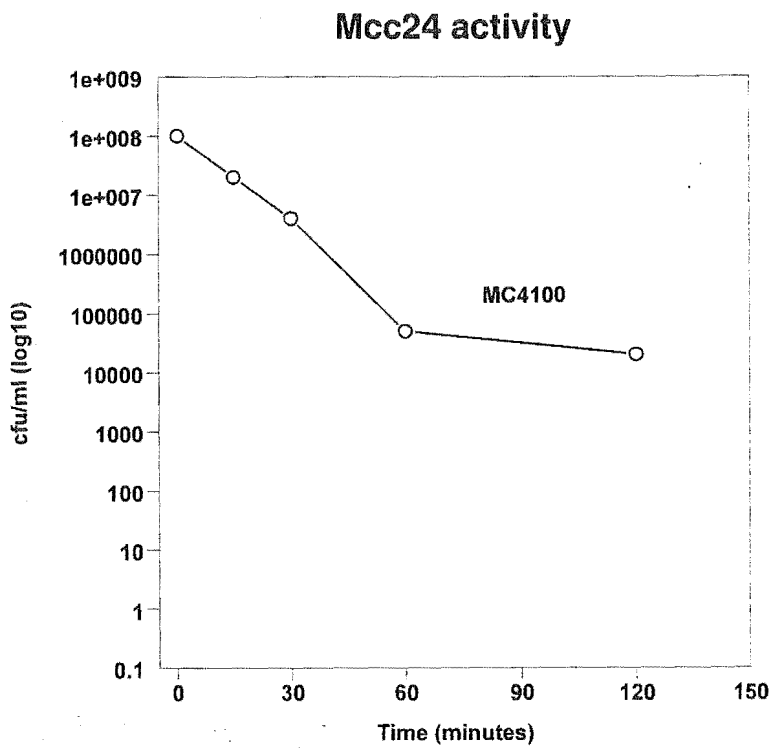


Figure 36. Time series of microcin 24 activity. An exponential culture of MC4100 was grown to an OD_{600} of 0.12. A series of six tubes containing 50µl of cells and an equal volume of Mcc24 extract were each incubated for varying times. The viable cell count was determined and plotted against time.

3.4.6 THE ANTIBIOTIC EFFECT OF MCC24 ON SENSITIVE CELLS.

Disruption of the proton motive force.

One of the primary mechanisms by which microcins inhibit the growth of sensitive cells is through disruption of the proton motive force (Table1). The uptake of amino acids is an active process and is very sensitive to disruptions in the proton motive force. By using radioactively labelled amino acids it is possible to measure the rate of their uptake in cells treated with microcin extracts in order to determine if the microcin affects the proton motive force of the cell (Pugsley and Oudega, 1987).

In order to determine whether Mcc24 affects sensitive cell by disrupting the proton motive force, microcin extracts were prepared from M63 medium (Methods 2.9.2) and the titre determined to be 128 A.U./ml. The amino acid uptake assay was

performed using MC4100 and a MC4100 Mcc24 resistant strain (Mcc24^R)without Mcc24 and then with Mcc24 added 5 min prior to the addition of ^{35}S-methionine/cysteine. Uptake of amino acids by MC4100 (Mcc24^R) was found to be less than that observed for MC4100 (Figure 37).

The reduced uptake of amino acids by MC4100(Mcc24^R) suggested that spontaneous resistance to Mcc24 might affect cysteine or methionine uptake. This was tested by generating spontaneous Mcc24^R mutants from *cys*$^-$ or *met*$^-$ parental strains (Methods 2.3.4) and testing the mutants for the ability to grow on M63 agar plates supplemented with cysteine (20μg/ml) or methionine (20μg/ml). Mcc24 resistant strains of *E. coli* JM246 (*cys*$^-$) and N3002 (*cys*$^-$) were found to grow on M63 agar supplemented with cysteine M63 but no growth was observed using M63 agar. Mcc24 resistant mutants of *E. coli* LE392 (*met*$^-$) and K802 (*met*$^-$) appeared to have reduced growth on M63+methionine compared to the parental strain, with neither growing on M63 agar. The two K802 Mcc24^R mutants were also ColV sensitive, however LE392 Mcc24^R #1 was ColV resistant. In order to quantify the difference in growth between *met*- parental and Mcc24^R strains, the experiment was repeated using 1ml of overnight culture washed in 1x M63 salts. Serial dilutions of the mutant and parental strains were found to grow equally well on M63 agar+methionine with neither growing on M63 agar, indicating that there was no real difference in growth.

Nuclease activity assay.

Nuclease activity is mechanism by which some E type colicins inhibit cell growth (Toba *et al.*, 1988), however no microcins have been found which function in this manner (Table 1). As a matter of interest Mcc24 extracts were prepared from MC4100(pGOB18) and MC4100(pGOB181::mini-Tn*10tet mtf*S$^-$) #2 (Mcc24$^-$, Results 3.11.2) as described in Methods 2.9.5. Nuclease activity of both extracts was tested on λ *Hin*dIII cut DNA and uncut pBR322 (Methods 2.10.8). The boiling and rapid cooling described in the method resulted in all the DNA becoming single stranded, producing the smearing observed in gel electrophoreses (Figure 38). Nuclease activity was found in the MC4100(pGOB18) extract, resulting in the linear and circular DNA being degraded over time, suggesting that Mcc24 functions as an endonuclease activity (Figure 38).

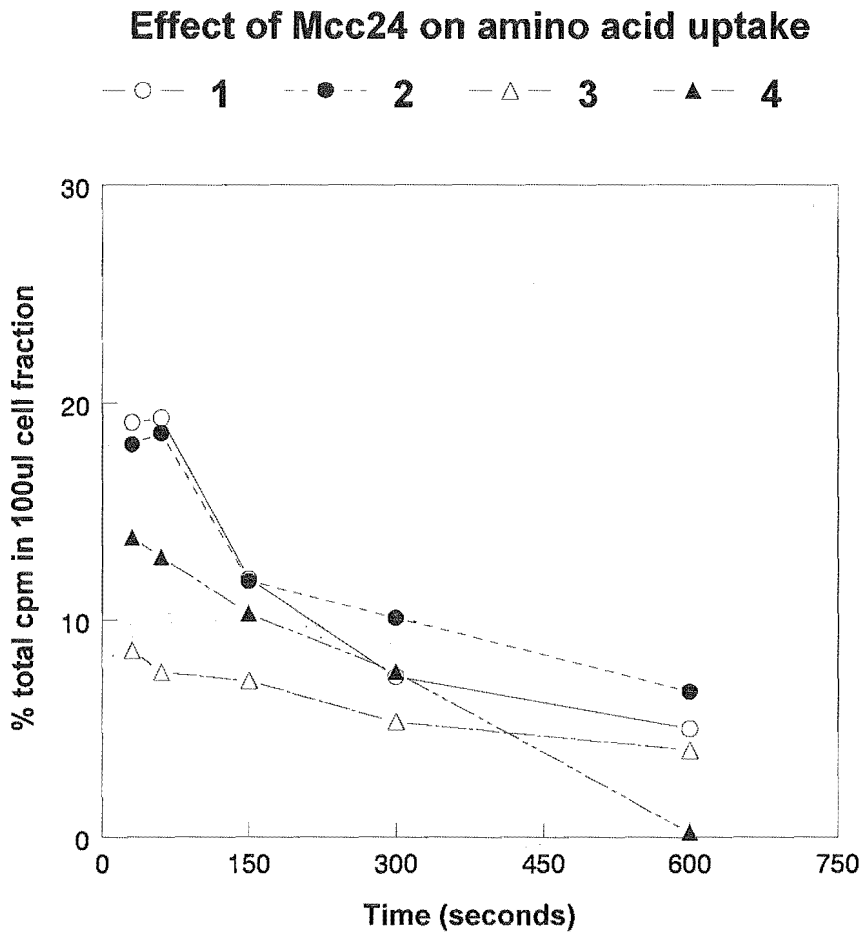


Figure 37. Effect of microcin 24 on amino acid uptake. Cultures of MC4100 and MC4100 (Mcc24^R) were grown to an OD_{600} of 0.15-0.2, washed and resuspended in M63 medium + Cm (25μg/ml). Aliquots of 1ml were incubated with Mcc24 extract for 5 minutes prior to the addition of ^{35}S-methionine/cysteine. Controls did not contain any Mcc24 extract. Samples (102μl) were taken at 30sec, 1min, 2.5min, 5min and 10min intervals and 2μl used to determine the cell concentration of the sample. The cpm of the cell fraction was determined using a Wallac 1410 Liquid Scintillation Counter and plotted as a percentage of the total cpm (cells + supernatant) against time. Treatments: 1, MC4100; 2, MC4100+Mcc24; 3, MC4100 (Mcc24^R); 4, MC4100 (Mcc24^R) + Mcc24.

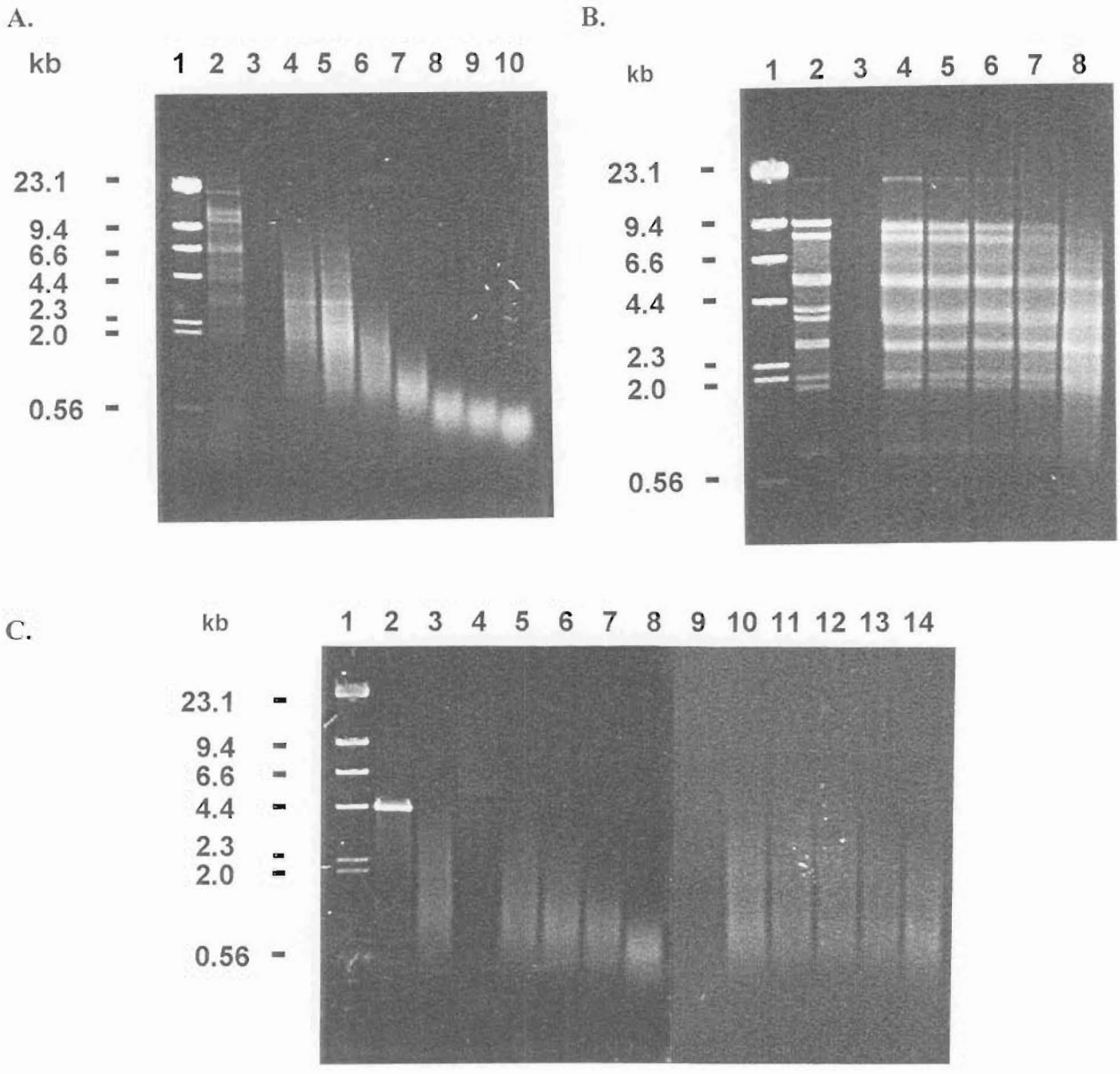


Figure 38. Nuclease activity test using microcin 24$^+$ and microcin 24$^-$ extracts. Protein extracts with Mcc24 activity (Mcc24$^+$) and without activity (Mcc24$^-$) were produced from MC4100(pGOB18) and MC4100(pGOB181::mini-Tn10 #2, mtfS$^-$) respectively. Samples were analysed by gel electrophoresis in a 0.6% agarose gel. **A.** Activity test using λ *Hin*dIII cut DNA and Mcc24$^+$ extract. Lanes: 1, λ *Hin*dIII standard; 2, λ *Hin*dIII without Mcc24$^+$ extract; 3, Mcc24$^+$ extract; 4-10, λ *Hin*dIII cut DNA incubated with Mcc24$^+$ extract for 5, 10, 15, 30, 60, 90 and 120 minutes respectively. **B.** Activity test using λ *Hin*dIII cut DNA and Mcc24$^-$ extract. Lanes: 1, λ HindIII standard; 2, λ HindIII without Mcc24$^-$ extract; 3, Mcc24$^-$ extract; 4-8, λ *Hin*dIII cut DNA incubated with Mcc24$^-$ extract for 5, 10, 15, 30 and 60 minutes respectively. **C.** Activity test using uncut pBR322. Lanes: 1, λ *Hin*dIII standard; 2, pBR322 linearised with *Eco*RI; 3, pBR322 without Mcc24$^+$ extract; 4, Mcc24$^+$ extract; 5-8, uncut pBR322 DNA incubated with Mcc24$^+$ for 5, 10, 15 and 30 mininutes respectively; 9, Mcc24$^-$ extract; 10-14 uncut pBR322 DNA incubated with Mcc24$^-$ extract for 5, 10, 15, 30 and 60 minutes respectively.

3.5 PROTEINS INVOLVED IN THE EXPRESSION, REGULATION AND ACTIVITY OF MICROCIN 24.

3.5.1 EFFECT OF TOLC ON MICROCIN 24 EXPORT.

Tol C had previously been shown to be involved in the export of ColV (Gilson *et al.*, 1990). Since the export of Mcc24 utilises proteins homologous to CvaAB as shown by trans-complementation (Results 3.1.3), it was of interest to determine if TolC was also involved in Mcc24 export.

The plasmid pGOB18 was electroporated into TolC mutant strains which had previously been used to assay the effects of TolC on ColV secretion and were designated "tight" (*E.coli* GC7459) and "leaky"(*E.coli* GC7442) (Gilson *et al.*, 1990). Transformants were assayed for microcin production using the patch and overlay test (Methods 2.3.3). Export of Mcc24 was absent in both TolC mutants and chloroforming failed to restore microcin activity. The presence of pGOB18 in both strains was confirmed by plasmid extraction and restriction analysis.

To determine if TolC was involved in the translocation of Mcc24 into sensitive cells, the patch test was performed using the "tight" and "leaky" TolC mutants as lawns. Both mutants were found to be Mcc24 sensitive.

3.5.2 EFFECT OF TONB ON MICROCIN 24 ACTIVITY.

TonB is another outer membrane protein that has been previously found to have a role in microcin import (Pugsley *et al.*, 1986; Chehade and Braun, 1988; Salomón and Farías, 1995). *E. coli* BzB1192 (*tonB*$^-$) was found to be sensitive to Mcc24 when used as a lawn in the patch test. Electroporation of pGOB18 into *E. coli* BzB1192 produced transformants which all produced Mcc24 to the same level as MC4100(pGOB18).

3.5.3 CHARACTERISATION OF THE MICROCIN 24 RECEPTOR ON SENSITIVE CELLS.

Pugsley and Oudega (1987) have produced a set of *E. coli* strains which contain mutations in the major microcin or colicin receptor genes. By using these strains as lawns in the patch test (Methods 2.3.3), it is possible to determine the receptor on sensitive cells responsible for Mcc24 binding.

Various strains producing Mcc24, ColV and other colicins or microcins were spotted onto lawns of the indicator strains (Table 7). One strain, PAP710 (*semA*::Tn*5*) was found to be resistant to Mcc24. The SemA protein is responsible for sensitivity to MccE492 (Pugsley *et al.*, 1986), therefore activity of MC4100(pGOB18) against *K. pneumoniae* RYC472 (MccE492$^+$) was confirmed by using RYC492 as a lawn in the patch test. MC4100(pGOB18) produced the same sized halo on RYC472 as on MC4100.

Table 7. Activity of microcin 24 against *E. coli* receptor mutants.

					Activity			
Strain	Genotype	Mcc 24[a]	Mcc 24[b]	Mcc E492	Col V	Col A	Col E1	MC4100
BzB1013	*fepA*	+	+	+	+	+	+	-
BzB1030	*butB*	+	+	+	+	-	-	-
BzB1190	*tsx*	+	+	+	+	+	+	-
PAP308	*ompF*	+	+	+	+	-	+	-
PAP1402	*ompR*::Tn*5*	+	+	+	+	-	+	-
PAP702	*ompA*	+	+	+	+	+		-
PAP710	*semA*::Tn*5*	-	small opaque	small opaque	+	+	+	-
MC4100		2mm	1mm	2mm	2mm	1mm	1mm	-

Strains: Mcc24[a], MC4100(pGOB18); Mcc24[b], 2424(p24-2); MccE492, RYC472; ColV, MC4100(pHK-11); ColA, BzB2101(pColA-CA31); ColE1, BzB2104(pColE1-K53). Production or absence of a halo is designated "+" and "-" respectively.

3.5.4 REGULATION OF MICROCIN 24 EXPRESSION.

Regulation by Fur.

The Fur repressor is a known regulator of gene expression in response to the iron status of the cellular environment and has been found to be regulate ColV expression (Chehade and Braun, 1988). In light of the similarities between the ColV and Mcc24 genetic systems, and the identification of a Fur box upstream of *mtfI* (Figure 24) it was of interest to determine the degree of Fur regulation in the expression of Mcc24.

Regulation by Fur was tested by electroporating *E. coli* GC4468 (*fur*$^+$) and *E. coli* GC4468 (Δ*fur*) strains with parental microcin plasmids p24-2(Mcc24$^+$) and pColV-K30 (ColV$^+$). These plasmids were used instead of recombinant plasmids in order to negate any effect plasmid copy number might have on gene expression. Transformants

were selected directly for microcin production, purified and grown overnight in LB broth. A 1µl aliquot of overnight culture was spotted onto a lawn of MC4100 on M63, LB and LB+2,2'-dipyridyl plates, incubated overnight and the halo size taken as a measure of Fur regulated microcin expression. The expression of ColV increased greatly when on plates with limited iron supply (M63 and LB+2,2'-dipyridyl), however the expression of Mcc24 increased only slightly in response to iron limitation (Figure 39).

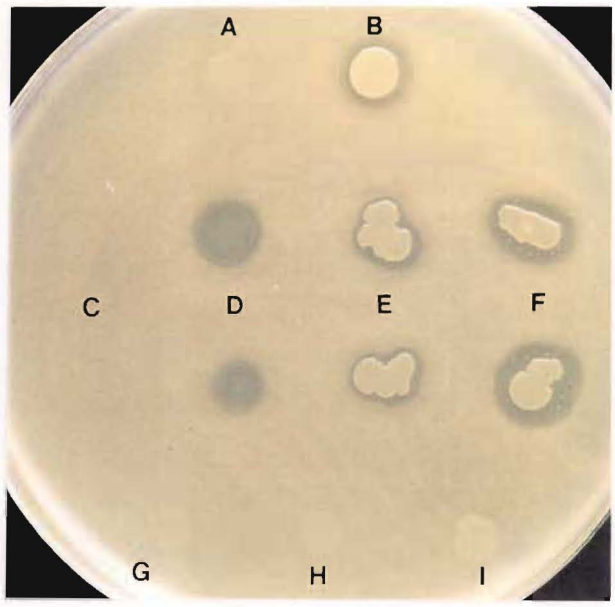
Regulation by σ^S and the possible involvement of *mdbA*.

The synthesis of microcins is known to occur when the cells enter stationary phase (Kolter and Moreno, 1993). Previously it had been found that the stationary phase sigma factor, σ^s encoded by the *rpoS* gene, was involved in the regulation of MccC7 production, and that derepression of an *rpoS* mutation could occur in an *hns*$^-$ background (Moreno *et al.*, 1992).

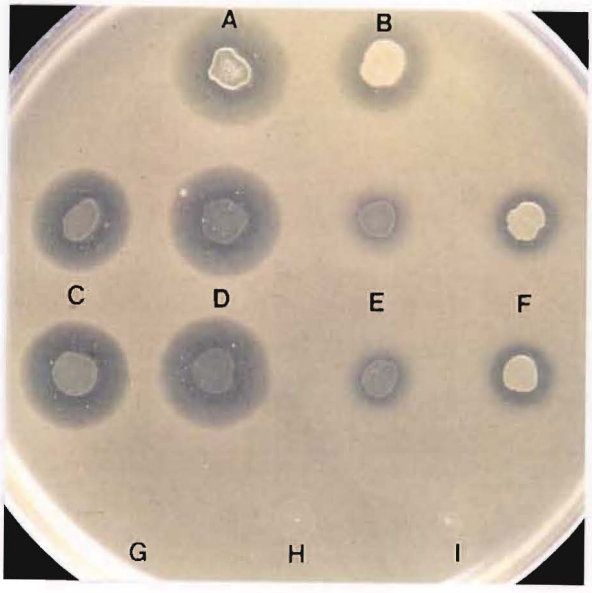
In order to determine if σ^s had a role in Mcc24 regulation, various plasmids were electroporated into *E. coli* ZB3(*rpoS*$^-$) and microcin production tested using the patch test at 37°C and 30°C and compared to that observed in a MC4100(*rpoS*$^+$) background. The constructs pGOB18 and pLOB813/pHK11-1 were both tested inorder to negate any effect of copy number since pGOB18 (pBR322 based) had a high copy number and pLOB813 (pACYC184 based) had a low copy number. No expression of Mcc24 occurred when pLOB813 was in the ZB3 background and expression from ZB3(pGOB18) was significantly reduced to that observed from MC4100(pGOB18) (Table 8).

To test if *mdbA* was also involved in *rpoS* regulated Mcc24 production by functioning in a similar manner to H-NS, pLOB813::mini-Tn*10tet* insertion #1 was also tested in both ZB3 and MC4100 backgrounds. The insertion point of this mutation had previously been determined by DNA sequencing to be after base 248 in the *mdbA* gene, producing a Mcc24$^-$, Mcc24 immune phenotype when complemented with pHK11-1 (Results 3.11.1). Expression of Mcc24 was found to be absent in both backgrounds, however at 30°C a very slight clearing was observed (Table 8).

1.



2.



3.

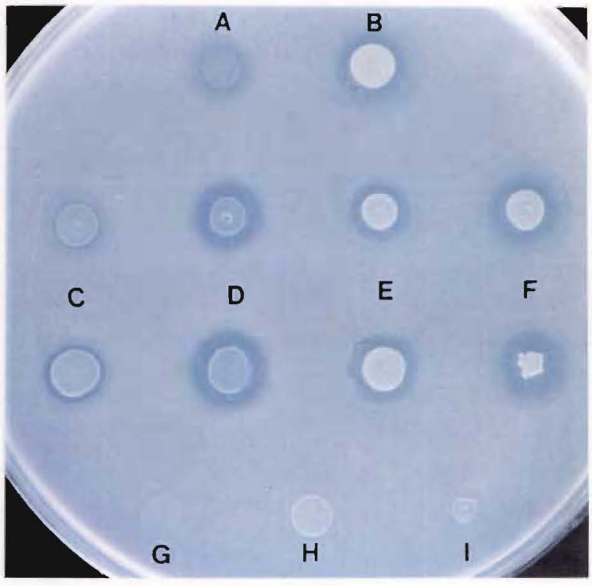


Figure 39. Regulation of microcin 24 expression by the Fur repressor. . Plates: **1**, LB plate. **2**, LB+2,2'-dipyridyl plate. **3**, MA plate.Strains: A, CSH26(pColV-K30::Tn*10* ColV$^-$); B, W3110(p24-2::Tn*10* #24 Mcc24$^+$); C, GC4468 *fur*$^+$(pColV-K30::Tn*10* ColV$^+$); D, GC4468 Δ*fur*(pColV-K30::Tn*10* ColV$^+$); E, GC4468 *fur*$^+$(p24-2::Tn*10* #24 Mcc24$^+$); F, GC4468 Δ*fur*(p24-2::Tn*10* #24Mcc24$^+$); G, GC4468 Δ*fur*; H, GC4468 *fur*$^+$; I, MC4100.

Table 8. Effect of *rpoS* on microcin 24 expression.

Strain	Plasmids	Temperature(°C) 37	30
MC4100			
	pGOB18	4mm	5mm
	pLOB813/pHK11-1	3mm	3mm
	pLOB813 no.1/pHK11-1	-	0.5mm opaque clearing
ZB3			
	pGOB18	3mm	3mm
	pLOB813/pHK11-1	-	-
	pLOB813 no.1/pHK11-1	-	0.5mm opaque clearing
MC4100		-	-
ZB3		-	-

Size of microcin halo on MC4100 lawn measured in mm from edge of colony to the edge of the clearing zone. Lack of microcin production denoted "-".

3.6 THE ROLE OF MCC24 IN DETERMINING THE VIRULENCE OF *E. COLI.*

The role that colicins and microcins play in the ecology of *E. coli* is thought to be one of defending the ecological niche of the producing strain against invading *E. coli* (Braun *et al.*, 1994) or to enhance the chance of faecal to oral transmission (Pugsley, 1984b). Resent evidence that ColV is directly cytotoxic to chicken embryos (Wooley *et al.*, 1994), suggests a more direct role for microcins in determining the virulence of *E. coli* strains.

To address this question two techniques were employed: a) testing Mcc24 activity against medically important bacteria; b) testing Mcc24 activity for toxicity against chicken embryos by expressing various recombinant Mcc24 encoding plasmids in MC4100, and analysing these strains using the embryo lethality assay.

3.6.1 ACTIVITY OF MCC24 AGINST MEDICALLY IMPORTANT BACTERIAL STRAINS.

The activity of Mcc24 against a number of medically important gram-positive and gram-negative bacteria was tested using the patch test. The plates were incubated at the appropriate temperature for the lawn bacteria and antibiotic activity checked after 6 and 16 hours. Mcc24 activity was found to be directed against gram-negative enteric bacteria strains (Table 9).

3.6.2 THE EFFECT OF MCC24 EXPRESSION ON E. COLI *VIRULENCE USING THE EMBRYO LETHALITY ASSAY.*

Strains of MC4100 harbouring the recombinant plasmid pGOB18 encoding the Mcc24 genes and mutant derivatives of this plasmid were sent to Prof. R.E. Wooley (University of Georgia) for trials using the embryo lethality assay (Wooley *et al.*, 1994). *E. coli* MC4100 harbouring the plasmids was not found to be virulent for the embryos (Table 10) and the embryos harvested on day six were found to be normal in appearance and of similar weights (R.E. Wooley, personal communication).

Table 9. Activity of microcin 24 against medically important bacterial strains.

		E.coli Colicin/Microcin producing strains											
	Morphology	1	2	3	4	5	6	7	A	B	C	D	E
Controls													
E.coli MC4100	bacilli	5	5	5	5	5	5	2	-	-	-	3	5
Salmonella typhimurium LT2	bacilli	5	3	5	5	5	-	-	-	-	-	-	-
Hospital Strains													
Gram positive													
Bacillus cerus	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
C. diphtheriae	bacilli	na	na	na	na	na	na	na	na	na	na	na	na
Listeria monocytogenes	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	cocci												
OX	"	-	-	-	-	-	-	-	-	-	-	-	-
BORSA	"	-	-	-	-	-	-	-	-	-	-	-	-
β Lactamase +	"	-	-	-	-	-	-	-	-	-	-	-	-
ORSA	"	-	-	-	-	-	-	-	-	-	-	-	-
MRSA	"	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus epidermidis	cocci	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus A	cocci	na	na	na	na	na	na	na	na	na	na	na	na
Streptococcus B	cocci	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus faecalis	cocci	-	-	-	-	-	-	-	-	-	-	-	-
Gram Negative													
Aeromonas hydrophila	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter	bacilli	2	1	4	4	4	1	3	-	-	-	-	4
Escherichia coli	bacilli	4	4	5	5	5	4	4	-	-	-	-	4
Hemophilus influenzae	bacilli	na	na	na	na	na	na	na	na	na	na	na	na
Klebsiella *	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
Neisseria meningitidis	cocci	na	na	na	na	na	na	na	na	na	na	na	na
Proteus mirabilis	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella typhimurium	bacilli	5	5	5	5	5	-	-	-	-	-	-	-
Serratia marcescens	bacilli	-	-	-	-	-	-	-	-	-	-	-	-
Shigella flexneri	bacilli	5	5	5	5	5	-	-	-	-	-	-	-
Shigella sonnei	bacilli	5	5	5	5	5	-	-	-	-	-	-	-
Yersinia enterocolitica	bacilli	2	2	-	-	-	-	-	-	-	-	-	-
Yeast													
Candida albicans		-	-	-	-	-	-	-	-	-	-	-	-

Strains: 1, *E.coli* 2424(p24-12 Mcc24$^+$); 2, *E.coli* 2424(p24-2 Mcc24$^+$); 3, *E.coli* MC4100(pGOB34 Mcc24$^+$);4, *E.coli* MC4100(pGOB342 Mcc24$^+$); 5, *E.coli* 71-18(pGOB420 Mcc24$^+$); 6, *E.coli* 2687, MccJ$^+$; 7, *E.coli* ZK126(pJC10 MccJ$^+$). Controls: A, MC4100; B, 71-18; C,ZK126; D, ZK126(pColV-K30 ColV$^+$); E, ZK126(pHK11 ColV$^+$). Colicin activity (edge of colony to edge of halo): -, no activity; 1, <0.5mm; 2, 0.5-1.0mm; 3, 1.0-2.5mm; 4,2.5-5.0mm; 5, >5.0mm; na, results not available because lawn did not grow. *, Mcc24 is known to be active against *Klebsiella pneumonia* RYC472 (Results 3.5.3).

Table 10. The effect of microcin 24 expression on the virulence of *E. coli* MC4100 using the embryo lethality assay.

Strains	Cfu/ml inoculated	Embryo deaths (/20)	Mean embryo weight (gm)	Bacterial titre post-inoculation(Log_{10} cfu/ml)
MC4100				
Group 1	356	0	20.3	7.43
Group 2	3560	0	16.7	7.48
MC4100(pBR322)				
Group 1	406	2	19.6	8.04
Group 2	4066	5	19.4	8.40
MC4100(pGOB18)				
Group 1	190	1	19.2	7.08
Group 2	1900	1	20.0	7.41
MC4100(pGOB181::mini-Tn*10* #2)				
Group 1	330	0	20.2	7.85
Group 2	3300	0	19.5	8.30
MC4100(pGOB181)	365			
Group 1	3650	0	18.4	8.32
Group 2		0	18.9	7.53
PBS		0	17.1	none
Uninoculated controls		0	20.6	none

Colony forming units (cfu) of strains in 0.1ml phosphate buffered saline (PBS) were inoculated into the allantoic cavity of 12-day old SPF embryos. Twenty embryos were inoculated per group. The number of embryo deaths was recorded over a six day period and the mean embryo weight for each group determined. At six days post-inoculation the bacterial titre within the allantoic fluid was determined.

CHAPTER 4.

DISCUSSION.

Bacteriocin production is a common phenomenon in a variety of bacterial species. Within the *Enterobacteriaceae*, the production of colicins and microcins has been frequently observed among natural populations (Pugsley, 1984b; Riley and Gordon, 1992; O'Brien *et al.*, 1996) and provides some of the most substantial evidence for a significant ecological advantage provided by these bacteriocins. To date only ColV has been found to have a role in pathogenicity, either by an association with virulence factors (Waters and Crosa, 1991) or directly through a cytotoxic effect (Wooley *et al.*, 1994).

Of the known microcins, only ColV, MccB17 and MccC7 have been studied in detail. The analysis of these microcins has indicated that this class of bacteriocin is more similar to the antibiotics produced by gram-positive bacteria than to colicins (Kolter and Moreno, 1992). Because of the paucity in the number of microcins analysed in detail, the evolutionary significance of these bacteriocins cannot be conclusively traced. Therefore further studies of novel microcins are needed in order to present a clearer picture of bacteriocin evolution and significance. The results presented in this thesis provide another example of a novel microcin, Mcc24, and contribute to the existing knowledge of this class of bacteriocin.

4.1 ESTABLISHMENT OF COLICIN 24 AS A TRUE MICROCIN, MICROCIN 24.

Previous classification of the antibiotic activity produced by *E. coli* 2424 based on the criteria used by Arsensio *et al.* (1976) for distinguishing between colicins and microcins, had determined that this activity was a colicin since diffusion through cellophane was not observed (O'Brien and Mahanty, 1994). SDS-PAGE analysis identified a protein of approximately 38kDa which was thought to be colicin 24 (O'Brien, 1992).

Prior to the DNA sequencing of the bacteriocin encoding region, all expression assays and SDS-PAGE gels were performed to maximise analysis based on the previous classification of this bacteriocin as colicin 24. However, when the CvaAB transport system was shown to export the activity encoded by pGOB18 (Results 3.1.3), there was a suggestion that this activity might be a microcin, since CvaAB is known to export another microcin, ColV (Gilson *et al.*, 1990). Subsequently, DNA sequencing failed to detect an open reading frame of sufficient size to encode a 38kDa protein within the structural region (Figure 25) as determined by trans-complementation (Figure 18), further indicating that the inhibitory activity had been wrongly classified. The possibility did exist however, that a colicin-sized molecule could be exported by an ABC transport system since CvaAB has been shown to facilitate the export of larger proteins (Fath *et al.*, 1991). In order to resolve the problems in classification of colicin 24, the cellophane test was repeated and showed that the activity encoded by pGOB18 was able to diffuse through the cellophane membrane (Figure 34). Once diffusibility through cellophane had been established, extraction and SDS-PAGE of the activity confirmed the presence of a peptide band corresponding to the 7.7kDa predicted by DNA sequencing (Figure 35). The proteinaceous nature of Mcc24 was confirmed by treatment with proteinase K, which inhibited the antibiotic activity (Results 3.4.3). As a result of the experimental evidence, the activity encoded by pGOB18 was re-named microcin 24 (Mcc24).

The reclassification of bacteriocins is not uncommon. For example, after detailed genetic analysis, ColV has also been identified as a microcin (Fath *et al.*, 1992). Previously in our laboratory it had been observed that the properties of cellophane change over time or after different sterilisation methods, which may account for some incorrect classifications. Different *E. coli* strains were also found to have different growth rates on cellophane, necessitating the use of the correct strain to harbor the plasmids in order to get sufficient expression of the microcin during the assay. The cellophane test therefore allows only a tentative classification of a novel bacteriocin and confirmation of this classification requires further genetic analysis.

4.2 GENETIC ORGANISATION OF THE MICROCIN 24 ENCODING REGION.

As described in the introduction, there are differences between the organisation of the operons encoding microcins resident in natural plasmids. The four ColV genes which form two converging operons (Figure 3) represent the simplest arrangement of genes for the production of peptide antibiotics. Microcins B17 and C7 have a more complex genetic system (Figures 4 and 7 respectively) which reflects the high degree of modification that occurs in the formation of these microcins.

In the case of Mcc24, several lines of evidence were combined in order to compare the Mcc24 genetic region with known systems and this evidence indicates that the Mcc24 genetic organisation has some similarities to that of ColV. First, the creation of various deletion derivatives from the original clone pGOB34 (O'Brien and Mahanty, 1994), identified important restriction sites and helped to define distinct genetic regions important in Mcc24 production. The *EcoRI* site joining the two *EcoRI* fragments was shown to be crucial for Mcc24 production since separating the fragments resulted in the loss of production (pGOB421 and pGOB423, Figure 10). However pGOB423 still conferred Mcc24 immunity.

It was hoped to determine gene boundaries and promoter locations within pGOB18 using mini-Tn*10lacZ* mutagenesis (Results 3.1.2). However, all of the Mcc24$^-$ insertions fell within the 3.44kb *Eco*RI-*Bam*HI fragment and Mcc24^{RED} insertions were located in the 2kb *Sau*3AI-*Eco*RI fragment suggesting that the latter fragment might be a structural or regulatory encoding region (Figure 13). By determining the β-galactosidase activity for each insertion , it was possible to show that the light blue phenotype observed for several insertions was the result of negligible activity, since Miller, (1972), defined full activity as 1000 Miller units and activity of approximately 1 Miller unit as negligible. Significant β-galactosidase activity was only observed when *lacZ* was transcribed left to right (Figure 13), suggesting that two or three promoters may exist within this region and transcribe in the same direction. Gilson *et al.* (1987) had previously been able to create mutations within the ColV encoding region and identify insertions within the transport genes. However, this technique failed to identify a transport encoding region for Mcc24 and will be discussed later (Discussion 4.3.1).

Previous analysis of ColV has shown that lysing cells by exposing them to chloroform vapour chloroform, is a useful technique for determining mutations in transport genes (Gilson *et al.*, 1987). This technique was not successful when analysing mutations in pGOB18, therefore the identification of functional units involved in Mcc24 production was obtained through DNA sequencing and trans-complementation. The gene of interest was sequenced using a *lacZ* primer and the sequence was compared to known sequences using the BLAST server. Sequence similarity comparisons to the ColV transporters CvaA and CvaB (Figure 14) identified a possible function for the gene disrupted by mini-Tn*10* insertion which could be confirmed by trans-complementation. Gilson *et al.* (1987) had already established that trans-complementation between genes cloned into compatible plasmids can be used to elucidate gene function. Utilising this property, it was possible to show that insertions pGOB18::mini-Tn*10* insertions #9, 14 and 18 were in transport genes since the loss of function could be replaced by CvaAB (Results 3.1.3, Figure 15).

Trans-complementation not only allowed gene functions to be determined, but was also of use in defining the boundaries between the Mcc24 genes. If a deletion derivative contained only the structural gene(s), complementation with CvaAB would restore the lost export function whereas no complementation would be observed between CvaAB and derivatives containing only the transport genes. This technique identifed pGOB181 and pGOB423 as encoding the Mcc24 structural and immunity genes (Results 3.1.5; Figure 17). The boundary between the two transport genes (1 and 2) was also determined to within 50bp by complementation (Figure 18). Of interest was the failure to obtain complementation between pGOB421/pLY21 and pLOB421/pGOB181. This indicated that a small portion of the transport genes encoding the promoter may be disrupted by the *Eco*RI site located between pGOB421 and pGOB423. As a result, pGOB421 and pLOB421 would encode incomplete transport genes. The sequence data from pGOB18::mini-Tn*10* #18 (Figure 14) identified a putative promoter in the first 50bp of the sequence, confirming that location of the promoter for the transport genes.

The final confirmation of the physical map for the Mcc24 encoding region was obtained by DNA sequencing (Results 3.2; Figure 25) and identified three operons, *mdbA*, *mtfI/mtfS* and *mtfA/mtfB* (Results 3.2.3). Although the genetic organisation of the Mcc24 encoding region most closely resembles that of ColV, the transcription of the Mcc24 operons in the same direction, and the identification of another open reading frame, *mdbA* located upstream of *mtfI*, indicates significant differences between the ColV and Mcc24 genetic systems. Microcins B17, C7 and the bacteriocins produced by gram-positive bacteria have a more complex genetic arrangement than Mcc24 (Moreno *et al.*, 1992, Gonzáles-Pastor *et al.*, 1995 and Jack *et al.*, 1995 respectively) which suggests that Mcc24 does not undergo the high degree of modification experienced by these bacteriocins.

Intergenic regions.

An interesting feature of the ColV genetic region is a 169bp intergenic region between *cvaC* and *cvaB* (Gilson *et al.*, 1990). The RNA encoded by this region can be folded into a highly stable structure, the significance of which is not known (Fath *et al.*, 1992). The genetic region encoding MccH47 also contains a large 3kb intergenic region between the *mchA* and *mchB* genes which does not appear to affect MccH47 production, even though a 43.5kDa protein is encoded by this region (Gaggero *et al.*, 1993). A 447bp intergenic region is located between the stop codon for *mtfS* and the transcription initiation codon for *mtfA* (Figure 24), however there is no evidence to suggest that this region is involved in Mcc24 expression. One insertional mutation within this region, pGOB18::mini-Tn10 #R3 (Figure 13), did result in a reduction in Mcc24 expression. This reduction could be due to the polar effect of the mini-Tn*10* insertion disrupting the transcription of the *mtfS* gene rather than affecting a regulatory mechanism. IS*10* insertions are known to have polar effects, disrupting gene expression (Iida *et al.*, 1983). A second intergenic region of 243bp exists between the end of *mdbA* and the start of *mtfI*. The only insertional mutations within this region are located within the P_1 promoter (Results 3.3.2) and so it is not thought that this intergenic region has a role in Mcc24 regulation except for the fact that the P_1 promoter is located within this region.

4.3 MICROCIN 24 PRODUCTION, EXPORT AND ACTIVITY.

4.3.1 THE STRUCTURAL GENE ENCODING MICROCIN 24.

Identification of *mtfS* and its protein product MtfS.

Through complementation, mutagenesis and protein analysis it was possible to confirm that the open reading frame identified through DNA sequencing as *mtfS*, was the structural gene encoding Mcc24. By growing MC4100(T14/pHK22-6) on LB + 2,2'-dipyridyl in order to allow a limited amount of cell growth, it was possible to visualise a small amount of Mcc24 production (Figure 30) identifying *mtfS* as the structural gene since no other open reading frames were encoded by this deletion. Insertional mutations within *mtfS* all produced a Mcc24$^-$ Imm$^+$ phenotype (Result 3.3.2), except insertions pLOB813::mini-Tn*10* # 2 and #3 (Figure 31). An effect of copy number on Mcc24 expression had previously been noted when analysing the gene *mdbA* (Discussion 4.3.4), and so it is possible that the reduced copy number of pLOB813 compared to pGOB181 resulted in the production of Mcc24 below the sensitivity limits of the patch test. These insertions also produced a poor growing phenotype which again would further reduce levels of Mcc24. Confirmation that *mtfS* encoded a protein was obtained by expressing the gene in minicells. The protein band of approximately 7.5kDa which was visualised (Figure 33, lane 2) is thought to be Mcc24 and is comparable to the protein band observed after extraction of Mcc24 from culture supernantants (Figure 35B). The nucleotide sequence of *mtfS* showed that the open reading frame consisted of 270bp, encoding a putative 9.42kDa (90 a.a.) protein (Figure 25). The disparity between the predicted and observed size of the protein, suggests that Mcc24 is the mature form of a post-translational modified precursor. Modification of the primary translation product is a common feature of peptide antibiotic biosynthesis in both gram-negative and gram-positive bacteria (Kolter and Moreno, 1992) and can range from cleavage of a leader sequence as seen for ColV (Fath *et al.*, 1994) to substantial amino acid modifications like that observed for MccB17 (Yorgey *et al.*, 1993). It is therefore possible, based on previous research, that Mcc24 is subjected to post-translational processing or modification.

Analysis of the *mtfS* DNA sequence and comparison to known peptide antibiotics.

The nucleotide sequence of *mtfS* did not show significant identity with protein sequences contained in the BLAST data base (Appendix 6). However upon close inspection of the sequence, a leader peptide was identified (Figure 27). This leader sequence was found to have significant similarity to the double glycine leader sequence identified in non-lanthionine containing antibiotics and the Group AII lantibiotics produced by gram-positive bacteria (de Vos *et al.*, 1995; Jack *et al.*, 1995), and ColV which is the only bacteriocin produced by gram-negative bacteria which is currently in this group (Fath *et al.*, 1994; Håvarstein *et al.*, 1994). Gilson *et al.* (1990) has identified the glycine residues at positions 14 and 38 as being critical for the export of ColV. Point mutations of Gly14 to Asn and Gly38 to Arg produced a leaky phenotype, however Gly14 to Asp resulted in an export deficient phenotype. Further analysis by Zhang *et al.*, (1995), has shown that the leader containing the Gly 14 to Asn mutation is not processed, whereas the Gly 14 to Asp mutation is incorrectly processed. The characteristic features that are present in double glycine leader sequences can all be identified within the MtfS leader sequence (Figure 27 and 29C). These features are: the conserved hydrophobicity and charged residues, a general hydrophilic nature especially in the N-terminus and GA, GS or GG at positions -2 and -1

Leader sequences are required for efficient export of the bacteriocin out of the cell, unlike the leader for MccB17 which is characteristic of the lantibiotic leader sequences and is required for maintaining protein solubility (Kolter and Moreno, 1992; Yorgey *et al.*, 1993). Export of the bacteriocin and removal of the leader sequence is mediated by the ABC transporter, which has a 100-150 amino acid N-terminal extension that contains the proteolytic domain (Håvarstein *et al.*, 1995). The export of MtfS by the ABC transporters MtfAB and CvaAB (Results 3.1.3), suggests that the MtfS leader peptide identified by DNA sequencing is functional, since the normal substrate for CvaAB, ColV, is also known to contain a double glycine leader sequence (Fath *et al.*, 1994). By analysing the export of ColV, Zhang *et al.*(1995) have shown that CvaB is sufficient for the processing of pre-ColV and that in the absence of CvaA and CvaB, pre-ColV is unstable in the cell. Protein analysis of MtfS and pre-MtfS using minicells has also shown that in the presence of MtfA and MtfB, pre-MtfS can be identified

(Figure 33, lane 2) and the size of pre-MtfS corresponds to the 9.42kDa predicted by nucleotide sequencing. In the absence of the transport proteins however, there does not appear to be any pre-MtfS (Figure 33, lane 3) suggesting that like pre-ColV, pre-MtfS is unstable in the absence of the exporters.

As mentioned earlier, one difference observed between secretion of ColV and Mcc24 is that the release of Mcc24 in transport deficient mutants cannot be restored by lysing the cell with chloroform. The activity of ColV however can be restored (Gilson *et al.*, 1987), even though the levels of activity are reduced due to the instability of internal ColV in export deficient strains (Fath *et al.*, 1992; Zhang *et al.*, 1995). Three possibilities exist which may explain this difference. Firstly it is possible that chloroform affected the activity of Mcc24. However, this was shown not to be the case (Results 3.4.4). The second possibility is that pre-MtfS is inactive, thereby preventing the formation of a halo upon lysis, since activation of the toxin requires removal of the leader sequence during export. Evidence for this possibility comes from the generation of immunity mutants in the presence of ABC transporters (Results 3.3.2), since the Imm$^-$ phenotype is not lethal to the producing cells. In contrast to this, Zhang *et al.* (1995) have shown that ColV is only toxic when presented to the inner membrane from the periplasmic side, indicating the function of ColV in disrupting the membrane potential. This suggests that for ColV, the possession of a leader peptide does not affect it's toxic activity since ColV cannot insert into the inner membrane from the cytoplasmic side. This difference between ColV and Mcc24 may reflect an inherent difference in their mechanism of activity. In the third scenario, the possession of a leader sequence could make pre-MtfS so unstable that it is degraded rapidly. The absence of pre-MtfS in export deficient derivatives supports this hypothesis (Figure 33, lane 3). The distinction between the last two alternatives awaits confirmation.

Analysing the mode of action of Microcin 24.

Determining how a peptide antibiotic inhibits the growth of sensitive bacteria is the major goal in the study of peptide antibiotics. The mode of action is also reflected in the physical characteristics of the bacteriocin. It was necessary to first extract and purify the peptide in sufficient quantities prior to testing its biological activity. The purification of non-lanthionine containing bacteriocins, although yielding highly

purified molecules, has resulted in low yields of approximately 20% (Jack *et al.*, 1995). The purification of active ColV had been facilitated by TCA precipitation from induced culture supernatant's (Fath *et al.*, 1994) however, this procedure was not successful when applied to Mcc24 purification (results not shown). Once it was confirmed that Mcc24 was a microcin, the correct conditions for expression were achieved by growing the culture in M63 medium and the bacteriocin extracted from the supernatant using nitrocellulose filters, a crude method previously demonstrated to be successful in some instances (Pugsley and Oudega, 1987). Although these extracts were not pure, the high activity demonstrated using the critical dilution test (Figure 35A) and by the reduction in the viable cell count of a sensitive culture within one hour of exposure to the microcin (Figure 36), did allow for further investigation into the activity of Mcc24. The reduction in Mcc24 activity after 60 minutes during the viable cell count was thought to be due to the toxin being consumed during the incubation period.

One of the most common mechanisms by which peptide antibiotics are known to inhibit bacterial growth is through disrupting the membrane potential of the target cells. This can be monitored by following the uptake of radioactivly labelled amino acids, a process which requires an intact membrane potential and is sensitive to disruptions in the inner membrane. The lantibiotics Pep5, nisin and subtilin, all form voltage-dependent channels in the inner membrane causing a rapid efflux of amino acids within thirty seconds after the addition of the lantibiotic to sensitive cells (Sahl and Brandis, 1983; Ruhr and Sahl, 1985; Schüler *et al.*, 1989; Sahl, 1994). The microcins ColV, MccD140 and MccE492 have also been found to affect membrane potential resulting in the inhibition of amino acid transport (Duro *et al.*, 1979; Yang and Konisky, 1984; de Lorenzo and Pugsley, 1985). In contrast however, Mcc24 did not significantly affect amino acid uptake into Mcc24 sensitive and Mcc24 resistant cells (Results 3.4.6). Hydropathy analysis of MtfS (Figure 29C) also does not predict any large hydrophobic domains potentially associated with insertion into the inner membrane, as found with other bacteriocins which insert into membranes, such as ColV (Fath *et al.*, 1992). Of interest was the observation that a Mcc24^{R} mutant of LE392(*met*$^{-}$) was also ColV resistant (Results 3.4.6). The significance of this is not known, but may suggest a common pathway for Mcc24 and ColV uptake which has yet to be identified.

Nuclease activity is another mechanism by which a number of colicins have been shown to inhibit the growth of sensitive cells, however no microcins to date have been identified with this mode of action. Endodeoxyribonuclease activity is displayed by colicins E2, E7, E8 and E9 and all share significant protein similarities (Toba *et al.*, 1988). Colicins E3, E4, E6 and Cloacin DF13 all possess endoribonuclease activity and share extensive protein identity (de Graaf and Oudega, 1986), but not with the endodeoxyribonuclease colicins (Cole *et al.*, 1985). The lack of sequence identity between MtfS and the nuclease colicins (Results 3.2.3) was at first, not unexpected. However, extracts of Mcc24 were found to degrade both linear and covalently closed plasmid DNA (Figure 38) indicating DNase activity. The absence of nuclease activity in extracts prepared from MC4100 harbouring pGOB181::mini-Tn*10* #2 (*mtfS*), confirmed that this activity was Mcc24 (Figure 38). The method outlined (Methods 2.10.8) reduces the double stranded DNA to single stranded DNA by boiling the sample after exposure to Mcc24 and quickly cooling it on ice. This results in the DNA smears observed in Figure 38, rather than discrete DNA fragments obtained in other studies using colicins with nuclease activity. Even though this method does not indicate discrete digestion of the DNA, DNase activity is still observed since the size of the DNA fragments within the smears are reduced over time.

4.3.2 IMMUNITY TO MICROCIN 24.

An important component of bacteriocin encoding systems is the specific immunity that protects strains from the bacteriocin they produce. ColV is unique among microcins in that only one gene, *cvi*, is sufficient to encode immunity to ColV (Fath *et al.*, 1992; Zhang *et al.*, 1995). However, for MccB17 and MccC7, several genes, including those involved in microcin transport, are responsible for immunity (Garrido, *et al.*, 1988; Gonzáles-Pastor *et al.*, 1995). Baquero *et al.* (1995) have also identified a chromosomal gene *sbmC*, which confers resistance to MccB17 when cloned into a high copy number vector. For the bacteriocins Pep5 and nisin produced by gram-positive bacteria, full immunity has been found to require expression of both the immunity and structural genes (Jack *et al.*, 1995). Immunity to Mcc24 appears to be encoded by one gene *mtfI*. The immunity encoded by this gene was confirmed by the creation of deletion derivatives which encoded immunity (Figures 10, 12 and 30) and through the generation

of insertional mutations which abolished Mcc24 immunity (Table 6). Minicell analysis also confirmed the predicted size of MtfI (11.43kDa, Figure 25), since a protein band at approximately 11.5kDa was identified and was found to be absent in an *mtfI* mutant (Figure 33, lanes 2 and 5).

Both ColV and Mcc24 appear to be unique among peptide bacteriocins in that the immunity and structural genes form an operon in which the immunity gene is transcribed first. For microcins B17 and C7, the specific immunity gene is located downstream of the structural gene (Figure 4 and Figure 7) and for MccH47 which is chromosomally encoded, the immunity gene is located upstream of the *mchB* gene within the 3kb intergenic region (Laviña and Gaggero, 1992). The difference in genetic organisation may reflect the limited degree of post-translational modification for ColV and Mcc24, which would result in an active molecule being formed almost immediately. The producing cells in the case of ColV and Mcc24 must have immunity to the microcins before the structural gene is translated. In contrast, the other microcins are subjected to a high degree of post-translational modification prior to the active microcin being generated and also utilise export as a second mechanism of immunity, allowing sufficient time delay for the immunity protein to be produced.

The actual mechanisms by which immunity is conferred on the bacteriocin-producing strain are of great interest and vary depending on the mode of action of the bacteriocin. The immunity proteins which act against either pore-forming colicins or some bacteriocins produced by gram-positive bacteria are located in the inner membrane and prevent pore formation (Géli and Lazdunski, 1992; Jack *et al.*, 1995) as seen with Cvi, which functions to block the activity of exogenous ColV at the periplasmic side of the inner membrane (Zhang *et al.*, 1995). A different mechanism of immunity exists for colicins with nuclease activity, where the immunity protein binds directly to the C-terminal catalytic domain of the colicin, thereby preventing activity (Jakes and Lazdunski, 1992; Wallis *et al.*, 1994). Specific mechanisms also exist such as McbG which provides specific immunity to endogenous MccB17 by preventing the steps leading to cell death after the inhibition of DNA synthesis (Herrero *et al.*, 1986; Garrido *et al.*, 1988) and MccE and MccF which provide immunity against MccC7 by acetylation of the antibiotic target site and sequestering MccC7 in the periplasm, respectively (Gonzáles-Pastor *et al.*, 1995). Two models can be proposed that account

for how MtfI provides immunity to the Mcc24 producing cell. In the first model, MtfI binds to either the target site for Mcc24 action or to Mcc24 itself, thereby inhibiting activity. This model would be consistent with the mechanism of immunity to other bacteriocins which function as nucleases. However, for Mcc24, immunity is not required to protect against endogenous microcin. MtfI has not been found to have sequence identity to the other nuclease immunity proteins which are all known to possess commonality in their amino acid sequence (de Graaf and Oudega, 1986) and may discount this mechanism of activity. Another possibility is that MtfI blocks or sequesters MtfS at the inner membrane, thereby preventing entry of the toxin into the cells, a mechanism observed for MccB17 and MccC7 immunity (Baquero *et al.*, 1995; Gonzáles-Pastor *et al.*, 1995). Evidence supporting this model comes from the hydropathy analysis of MtfI which shows three hydrophobic regions (Figure 29B). These domains could be indicative of integral membrane domains and may imply that MtfI is associated with the inner membrane and function to block or sequester active Mcc24.

4.3.3 EXPORT OF MICROCIN 24.

The mechanism by which peptides are exported across membranes, and in particular the double membrane of gram-negative bacteria, has become an intense area of study. A common feature of double glycine leader peptide-containing peptides such as the lantibiotics subtilin and epidermin and the microcins B17 and C7 is that they are all exported in an energy dependent fashion by an ABC transporter complex (Garrido *et al.*, 1988; Fath *et al.*, 1994; Håvarstein *et al.*, 1994; Gonzáles-Pastor *et al.*, 1995; Håvarstein *et al.*, 1995). The first indication that Mcc24 was also exported by an ABC transport complex came from DNA sequence comparisons and trans-complementation experiments using the ColV transporters CvaA and CvaB with pGOB18::mini-Tn*10lac* insertions and various deletion derivatives (Results 3.1.3 and 3.1.5). Of interest, was the finding that complementation between the ColV and Mcc24 export systems was reciprocal, even though there was a slight reduction in ColV export using the Mcc24 exporters, as seen in Figure 19B and C, where plate B shows ColV production on a $Mcc24^R$ lawn, and plate C shows Mcc24 production on a $ColV^R$ lawn. No complementation was found between the *S. entomphila* chitinase transporter and the

Mcc24 transporters (Results 3.1.3), even though complementation between CvaAB and the *S. entomphila* chitinase transporter is known to occur (Shum, 1992). Fath *et al.* (1991) have previously shown that trans-complementation between different ABC transporters does occur. However, complementation is not necessarily reciprocal, presumably because of different specificities in export signals within each export system. This may also explain the differences in complementation observed between CvaAB, the Mcc24 transporters and the *S. entomphila* chitinase transporter. The reduced export of ColV when using the Mcc24 exporters may be due to differences in the leader sequences which, though similar (Figure 27), may have sufficient differences to reduce substrate recognition by the export apparatus.

The genes and their protein products involved in the export of Mcc24, have been found to have a very high degree of similarity in size and function to the CvaA and CvaB proteins required for ColV export. The predicted size of MtfA, 414 amino acids long (47kDa, Figure 25), is comparable to CvaA (47kDa) (Gilson *et al.*, 1990) and MtfA also has 71% identity and 86% positive identity to CvaA over the entire length of the protein (Appendix 6). This sequence similarity along with the complementation (Results 3.1.6 and3.2.4) identifies MtfA as a member of the MFP protein family which includes CvaA (Skvirsky *et al.*, 1995). The hydropathy profile of MtfA (Figure 29D) also follows the recognised pattern for MFP proteins with a N-terminal hydrophobic segment which may anchor the protein to the cytoplasmic membrane, a hydrophilic central region which spans the periplasm and a hydrophobic C-terminal region which forms a highly conserved outer-membrane domain (Dinh *et al.*, 1994). An interesting feature of the *cvaA* gene is an internal in-frame methionine codon which produces the truncated CvaA* protein (27kDa) (Gilson *et al.*, 1990). Analysis of the *mtfA* gene products using minicells has identified a protein band at approximately 47kDa (Figure 16, lane 2) which is missing in the *mtfA*⁻ mutants pGOB18::mini-Tn*10lac* #18 and 9 (Figure 16, lanes 3 and 4) and is thought to be MtfA. However unlike *cvaA*, no truncated form of MtfA was visible (Figure 16, lane 2) even though an internal ATG codon exists in the DNA sequence between bases 2341-2343 (Figure 24) which could give rise to a 27kDa protein. This suggests that unlike CvaA, there is only one form of MtfA present within the cell.

The *mtfB* gene is predicted to encode a 707 amino acid protein (79kDa, Figure 25) which has been found to have 73% identity and 87% positive similarity to CvaB as well as a high degree of similarity to other ABC transporters (Appendix 6), indicating that MtfB is a member of the ABC transporter family of proteins. Confirmation of the predicted size of MtfB was not possible using minicell analysis, however CvaB and HylB have also not been visualised by SDS-PAGE because of the low levels of protein expression and the difficulty in characterising complex integral membrane proteins (Fath *et al.*, 1992). The region of strongest similarity among ABC transporters is the 200 amino acid ATP-binding domain (Higgins *et al.*, 1986), comprising the two Walker motifs A and B (Walker *et al.*, 1982) and a linker peptide (Koronakis *et al.*, 1995). These features have all been identified within MtfB (Figures 24 and 28) and are located within the region of highest similarity to known ABC transporters as determined using the BLAST server (Appendix 6). Although the binding of ATP by MtfB has not been determined experimentally, trans-complementation between MtfAB and CvaAB (Results 3.1.3 and 3.1.6) suggests that the two systems are homologous and predicts that MtfB must bind ATP, since the export of ColV is dependent on the hydrolysis of ATP by CvaB. ABC transporters which export peptides with a double glycine leader sequence, are also thought to encode a proteolytic domain in their N-terminal region which is responsible for cleavage of the leader sequence (Håvarstein *et al.*, 1995). MtfB is of a similar size to that of other double glycine peptide transporters and shares a high degree of identity to CvaB within this N-terminal region, suggesting that MtfB also contains a proteolytic domain that cleaves the leader sequence from pre-MtfS during export. Like CvaAB which have been found to be required for the processing of pre-ColV during export (Zhang *et al.*, 1995), MtfAB also appears to be required for pre-MtfS stabilisation and processing, since pre-MtfS is inactive in *mtfAB* deficient strains (Results 3.1.2) and is absent from the protein profiles of minicells in a *mtfAB* deficient background (Figure 33, lane 3).

Another similarity between the ColV and Mcc24 export systems is the requirement for TolC, which was previously found to be necessary for the export of α-hemolysin and ColV across the outer-membrane (Wandersman and Delepelaire, 1990; Gilson *et al.*, 1990). TolC was also found to be required for the secretion of Mcc24 however, unlike the secretion of ColV which is termed "leaky" in some *tolC* mutations

(Gilson *et al.*, 1990), the expression of Mcc24 from the *tolC* strains was absent even after exposure to chloroform vapour (Results 3.5.1). This suggests that there are subtle differences in the export of ColV and Mcc24 as previously suggested by complementation, and that activation of Mcc24 requires the export process to be completed.

4.3.4 REGULATION OF MICROCIN 24 EXPRESSION.

Promoters controlling Microcin 24 expression.

Putative regulatory promoter elements controlling of the expression of the Mcc24 genes were identified from the DNA sequence information (Figure 24) and the functionality of these elements was confirmed by mutagenesis and trans-complementation. The promoter $P_{mdb,}$ is thought to regulate the expression of *mdbA,* and has sequences similar to those required for transcription using $E\sigma^S$. This may provide a mechanism by which Mcc24 expression is growth-phase regulated since *mdbA* is required for the production of Mcc24. The expression of *mtfI/mtfS* is controlled by the P_1 promoter located upstream of the *mtfI* gene (Figure 24 and 25). Sequences with a high degree of similarity to σ^{70}-dependent promoters are found within the P_1 DNA sequence suggesting that transcription from this promoter is mediated by the $E\sigma^{70}$ holoenzyme. Mutations within this region significantly reduced the production of Mcc24 and abolished immunity to Mcc24 as seen with pGOB18 mini-Tn*10* insertions #R5 and R7 (Figure 13) which are located within the -35 region of the promoter (Table 5) and mini-Tn*10* insertions into pLOB813 and pGOB181 within this promoter (Results 3.3.2), which even on a low copy number plasmid still retain some Mcc24 expression (Table 6). The low level expression of Mcc24 in the P_1 promoter mutants indicates that a second promoter, P_2 which contains sequences with significant similarity to the σ^{70}- and σ^S-dependent promoter sequences, may function to produce low levels of Mcc24 (Figure 25). This was confirmed by the observation that the deletion derivative T14 was still able to produce some Mcc24 even in the absence of P_1 (Figure 30), suggesting that P_2 maybe responsible for low level transcription of *mtfS* before the full transcription of *mtfS* is initiated from P_1 in response to environmental signals. Another promoter which also has sequences similar to those observed in both σ^{70}- and σ^S-dependent promoters is

thought to regulate expression of the transport genes. As previously mentioned, the absence of this promoter resulted in lack of trans-complementation between pGOB421 or pLOB421 and pLY21. It is possible that transcription from P_2 and P_{AB} is mediated by both $E\sigma^{70}$ and $E\sigma^{S}$ since the binding of both holoenzymes to similar DNA sequences has been previously demonstrated (Tanaka *et al.*, 1993).

In comparison to the genetic arrangement of other microcins, the four promoters controlling Mcc24 expression are unique. The regulated expression of the MccB17 and MccC7 encoding regions is mainly controlled by a single promoter, P_{mcb} and P_{mcc} respectively (Moreno *et al.*, 1992). Also among the majority of microcins, for example ColV (Gilson *et al.*, 1990), MccC7 (Gonzáles-Pastor *et al.*, 1995), and MccH47 (Gaggero *et al.*, 1993), the direction of transcription of the genes involved in microcin production can vary. However, for MccB17 (Moreno *et al.*, 1992) and Mcc24, all the genes are transcribed in the same direction. The MccB17 encoding region also has a promoter within the main operon, a situation found in Mcc24 where the P_2 promoter occurs within the *mtfI/mtfS* operon.

The role of *mdb*A.

The gene *mdbA*, which was located upstream of the Mcc24 genes (Figure 24 and 25), is unique to the Mcc24 system since no other microcins are known to be positively regulated by a *cis*-acting element. Trans-complementation between pLOB813::mini-Tn*10* #1 and pHK11-1 (*cvaAB*$^{+}$), has shown that *mdbA* is required for the expression of Mcc24 since this insertion abolishes Mcc24 activity but retains immunity to Mcc24 whereas pLOB813/pHK11-1 expresses Mcc24 fully (Results 3.3.2) This contrasts with pGOB18::mini-Tn*10lac* #R2 (Results 3.1.2), where the insert is located between bases 352 and 353 (Table 5) producing a slightly reduced Mcc24 halo. By using *E.coli* ZK762, a strain carrying the plasmid copy number reducing mutation *pcnB* (Lopilato *et al.*, 1986), as the host strain for this plasmid, it has been possible to show that reducing the copy number the plasmid results in negligible expression of Mcc24 (M. Kamiya, personal communication). however). This suggests that the expression originally observed was a result of the high copy number of pGOB18, a pBR322 derivative.

Protein sequence comparisons and proposed mechanism of activity.

Interestingly, comparisons of the protein encoded by *mdbA* to known protein sequences using the BLASTX data base identified a significant region of identity between amino acids 15 to 86 of MdbA and other H-NS and H-NS-like proteins (Results 3.2.4). The first 90 amino acids of H-NS, the region with similarity to MdbA, has previously been shown to have 22-29% identity to structural protein such as myosin, resulting in the speculation that this region is involved in the oligomerisation of H-NS along the DNA (Ussery *et al.*, 1994). The hydropathy profile of MdbA (Figure 29A) shows that residues 43-86 form a highly hydrophilic domain, suggesting that MdbA functions as a homodimer and that this N-terminal domain is involved in protein-protein interaction between the MdbA monomers. Mutational analysis has shown that this domain is important, since pLOB813::mini-Tn*10* #1 and pGOB18::mini-Tn*10lac* #R2, insert within the codons for amino acids 52 and 87 respectively, disrupting this putative dimer-forming domain of MdbA. Evidence that MdbA functions as a dimer is seen in the minicell analysis, where the MdbA protein was confirmed as a 19.94kDa protein (Figure 16, lanes 2-8; Figure 33, lanes 2-5) which is absent in the *mdbA* insertion mutant (Figure 16, lane 9; Figure 33, lane 6). Also visualised in Figure 16 (lanes 2-8) and Figure 33 (lanes 2-5) is a 40kDa protein which is not present in lanes 9 and 6 respectively, and is thought to be a dimeric forms of MdbA. Figure 33 (lanes 2-5) also shows a 44kDa protein which is thought to represent an isomer of the 40kDa dimeric MdbA, since different species of H-NS have been identified by Spassky *et al.* (1984) and exist in varying ratios depending on growth conditions. The 44kDa protein oberved in figure 16 (lanes 2-8) is also present in lane 9 (*mdbA*$^-$) and therefore cannot be MdbA. The identification of this protein has yet to be determined.

Recently, H-NS has not only been seen as a component of the bacterial nucleoid (Drlica and Rouviese-Yaniv, 1987), but has also been implicated in gene regulation through altering DNA topology (Hulton *et al.*, 1990). The highly conserved amino acid sequence of approximately 135 residues, which is not too dissimilar to the size of MdbA (171 residues, Figure 25), confirms the ubiquitous nature of this protein (Ussery *et al.*, 1994). However, unlike typical DNA binding proteins which have a helix-turn-helix motif (Pabo and Sauer, 1984; Brennan and Mathews, 1989), H-NS utilises a stretch of

positively charged amino acids toward the C-terminus of the protein which are thought to be involved in DNA binding (Ussery *et al.*, 1994) and binds to DNA at regions containing curved DNA sequences (Zuber *et al.*, 1994). These curved DNA sequences are typified by having a rich AT content (Crothers *et al.*, 1990). Regions with a high AT content can also be found along the nucleotide sequence of pGOB18 (Figure 24), especially upstream of the *mtfS* and *mtfA* genes. The significance of these regions in relation to MdbA binding has yet to be determined, however it is tempting to speculate that MdbA acts in a similar fashion to H-NS and binds to these AT rich regions. MdbA binding would therefore regulate the expression of MtfS and also the expression of the transport genes which have been implicated in the post-translational modification and stabilisation of pre-MtfS (Discussion 4.3.3).

The regulation of microcin expression by H-NS has been previously determined for MccB17 and MccC7, however unlike Mcc24 which is positively regulated by MdbA, H-NS functioned as a repressor in the case of MccB17 and MccC7 (Moreno *et al.*, 1992). Positive regulation by the DNA binding protein, IHF has been demonstrated for MccB17 synthesis (Moreno *et al.*, 1992). Of interest, is the observation that the expression of MccC7 is de-repressed in an *rpoS* background when a *hns* mutation was introduced. In an attempt to identify a similar function for MdbA, pLOB813::mini-Tn*10* #1 (*mdbA*⁻) was expressed in *E. coli* ZB3 (*rpoS*⁻) (Results 3.5.4). The results were not conclusive, possibly because of *hns* encoded in the chromosome which may have interfered with the assay by substituting for MdbA. By performing the assay at 30°C in order to allow gene expression before the cells reached stationary phase, a faint clearing zone was observed (Table 8). However, this experiment needs to be repeated before any conclusions can be drawn suggesting a similar mechanism of activity between MdbA and H-NS. MdbA also has sequence identity to the *E. coli* StpA protein, which is an H-NS-like protein implicated in H-NS-independent gene regulation through binding RNA (Zhang *et al.*, 1996). Several mechanisms can be suggested by which MdbA could activate Mcc24 expression. Activation of Mcc24 could occur directly through MdbA binding the DNA or RNA encoding Mcc24, changing the configuration of the template into a transcriptionally or translationally competent state. MdbA may also repress/activate the transcription or translation of a chromosomally encoded repressor or activator, through binding to the DNA or mRNA templates encoding this, as yet,

undetermined molecule. The exact mechanism of MdbA regulation awaits experimental investigation.

A significant finding in this study is that the gene encoding MdbA is located on the same plasmid as the Mcc24 genes. If MdbA does function as a H-NS-like molecule, this would be the first report of such a protein being encoded on a plasmid, since all other H-NS-like proteins are chromosomally encoded (Drlica and Rouviere-Yaniv, 1987; Ussery *et al.*, 1994). The regulatory proteins involved in the biosynthesis of microcins B17, C7 and ColV are also encoded on the chromosome (Moreno *et al.*, 1992; Chehade and Braun, 1988). MdbA therefore represents a new class of microcin regulatory protein which is encoded as a *cis*-acting element, affecting the transcription and/or the translation of closely linked genes.

Regulation at stationary phase.

One of the interesting aspects of microcin expression is the induction of synthesis when the producing cells enter stationary phase. Both MccB17 and MccC7 are growth-phase dependent, however only MccC7 is regulated by *rpoS* (Diaz-Guerro *et al.*, 1989), which encodes σ^S the stationary phase sigma factor (Loewen and Hengge-Aronis, 1994). The expression of MccB17 from P_{mcb} is σ^{70}-dependent, utilising "gear-box" promoter sequences for transcription (Bohannon *et al.*, 1991). Like that of MccC7, the expression of Mcc24 is dependent on *rpoS*, since Mcc24 expression was repressed in an *rpoS*$^-$ background, however the effect was only observable when the genes encoding Mcc24 were present on a low copy plasmid such as pACYC184 (Results 3.5.4). As previously mentioned, DNA sequencing has identified promoter sequences with identity to σ^S and σ^{70} consensus sequences however, no gearbox sequences have been identified (Figure 24).

As part of a global regulatory system, other transcription factors have been found to act independently or in combinations to affect the transcription of σ^S-dependent genes. This may also be the case with Mcc24 expression since putative consensus sequences for σ^S are located within P_{mdb} and may result in the production of MdbA during stationary phase and subsequent induction of Mcc24 biosynthesis. Barth *et al.* (1995) have previously identified H-NS as a component of the *rpoS* regulatory network. In this study, the similarities between H-NS and MdbA may indicate that

MdbA is also part of this network. Of the three classes defined for σ^S-dependent genes with respect to H-NS (Barth *et al.*, 1995), the expression of MccC7 fits into the type two model in which the genes are more strongly expressed in an *hns*⁻ *rpoS*⁻ background than in an *rpoS*⁻ background alone. The expression of Mcc24 appears to fit into the type three model in which gene expression is weakly increased in an *hns*⁻ mutant during exponential growth, with H-NS acting as a positive activator for stationary phase growth as well as additional factors besides σ^S. In this scenario, MdbA would replace H-NS as the positive activator. Another mechanism by which Mcc24 expression is controlled may be through the regulation of the transport genes *mtfA* and *mtfB*, as discussed previously (Discussion 4.3.3). Consensus sequences for σ^S binding can be found within the *mtfA* promoter, therefore expression of the transport genes may be induced during stationary phase resulting in the processing of the unstable pre-MtfS. The P_2 promoter of *mtfS* also has σ^S consensus sequences which would be required to link the transcription of *mtfS* and *mtfAB*. As previously mentioned, the promoter sequences for P_1, P_2 and P_{AB} may also bind σ^{70} however, the lack of well defined promoter sequences within σ^S-dependent promoters can be compensated for by DNA bending (Espinosa-Urgel and Tormo, 1993). The AT rich regions located upstream of the *mtfS* and *mtfA* genes may indicate that these promoters preferentially bind σ^S, resulting in stationary phase dependent expression from P_1, P_2 and P_{AB}. These regions may also be the sites of interactions between MdbA and σ^S since both H-NS -like proteins and σ^S preferentially bind to curved DNA sequences.

Regulation by the Fur repressor.

Superimposed upon the regulation of Mcc24 expression by MdbA and σ^S is another global regulatory protein, the Fur repressor, which links gene expression to iron limitation in the environment. A putative Fur-box within the -35 promoter region of the P_1 promoter was identified by DNA sequencing and shares significant identity to the Fur-box consensus sequence (Figure 26), suggesting that Fur represses the transcription of both *mtfI* and *mtfS* from this promoter.

Included among the virulence genes regulated by Fur or Fur homologues, are the genes encoding the shiga-like toxin (Calderwood and Mekalanos, 1987), the shiga toxin, diptheria toxin and the *P. aeruginosa* exotoxin A (Litwin and Calderwood, 1993), as

well as ColV (Chehade and Braun, 1988). The Fur-box regulating ColV expression, 151-GATAATCATTATC-171 is, like that of Mcc24, located upstream of the microcin immunity gene *cvi* (Gilson *et al.*, 1990), and although shorter than the consensus sequence, it still contains the dyad repeat. Fur regulated expression of ColV is very strong, with expression in the presence of iron being significantly reduced and derepression occurring in iron limiting conditions or in Δ*fur* strains (Figure 39). The increase in Mcc24 expression in similar conditions was only slight compared to the expression of Mcc24 on LB media (Figure 39), suggesting that Fur has a minor role in regulating Mcc24 expression. Compan and Touati (1993) have shown that the expression of manganese superoxide dismutase in *E. coli* is Fur regulated, however Fur is involved in fine tuning expression to environmental signals and functions in conjunction with other regulatory molecules. Fur regulated expression of Mcc24 may also function to fine tune the expression of Mcc24 as part of a further global regulatory mechanism, possibly involving MdbA and σ^S.

4.3.5 MICROCIN 24 UPTAKE INTO SENSITIVE CELLS.

In order for microcins and colicins to be taken up by their target cells, they must first bind to an outer-membrane receptor and then be translocated across the outer and inner membranes by a second protein (Pugsley , 1984a). Both colicins and microcins have been shown to utilise the same receptors and translocation machinery, for example Colla, Ib and ColV all use Cir for a receptor and TonB as part of the translocation machinery (Pugsley, 1984a; Chehade and Braun, 1988). By using the microcin and colicin resistant or tolerant *E. coli* strains in the colicin reference collection (Pugsley and Oudega, 1987), it was possible to identify the gene encoding the outer-membrane receptor for Mcc24 binding as *semA* (Table 7). The only previously reported function for *semA* was as the gene encoding the receptor for MccE492 (Pugsley *et al.*, 1986). No cross immunity was found between *K. pneumoniae* RYC472(MccE492$^+$) and *E. coli* MC4100(pGOB18 Mcc24$^+$) indicating that they were in fact different microcins (Results 3.5.3). Uptake of MccE492 is mediated by TonB and ExbB, however Mcc24 activity was still found against strains carrying a *tonB* mutation (Results 3.5.2). Although MccE492 and Mcc24 share the same outer-membrane receptors, differences in their translocation appear to exist. This has also been demonstrated for MccB17 and

MccC7 which share the same outer-membrane receptor OmpF (Liu, 1994; Gonzáles-Pastor *et al.*, 1995), but utilise different inner-membrane proteins for translocation. The inner-membrane protein for MccC7 translocation has yet to be identified (Gonzáles-Pastor *et al.*, 1995). Differences in the target site between Mcc24 and MccE492 may be responsible for the utilisation of different translocation proteins because MccE492 depolarises the cell membrane and would not require entry into the cytoplasm (de Lorenzo and Pugsley , 1985), unlike Mcc24 which appears to have nuclease activity (Results 3.4.6).

4.3.6 THE PROPOSED MODEL FOR THE BIOLOGICAL ACTIVITY OF MICROCIN 24.

The results presented in this thesis have allowed a model to be proposed for the biological activity of Mcc24 (Figure 40).

4.4 SIGNIFICANCE OF MICROCIN 24 PRODUCTION.

The role of bacteriocin production in determining the virulence of *E. coli* is an important area of investigation in bacterial ecology. ColV is the only microcin which has been directly associated with virulence in *E. coli* (Smith, 1974; Smith and Huggins, 1976; Wooley *et al.*, 1994). An attempt to repeat the experiment of Wooley *et al.* (1994) using *E. coli* strains harbouring Mcc24 producing plasmid derivatives did not show any effect on the mortality rates of chicken embryos (Table 10). A significant difference between the original experiment using pHK11 and this trial lies in the origin of the parental Mcc plasmids pColV-K30 (ColV) and p24-2 (Mcc24). The plasmid pHK11 is derived from pColV-K30, a plasmid first extracted from *E.coli* K30, a chicken isolate however, pGOB18 is derived from p24-2 harboured by *E. coli* 2424, a human uropathogenic strain (O'Brien and Mahanty, 1994). Colicin V production has also been found to be significantly higher among *E. coli* isolates of animal origin than among *E. coli* isolates of human origin (Smith, 1974; Smith and Huggins, 1976). The difference in the origins of the microcin plasmids may affect the sensitivity of the assay in determining the direct toxicity of the bacteriocins. The chicken embryo lethality assay may be appropriate in assessing the significance of bacteriocins encoded by plasmids from *E. coli* of avian origin, however this assay may not be appropriate for microcins

encoded by plasmids from *E. coli* derived from human sources. To assess the sensitivity of the embryo lethality assay to bacteriocins produced by bacterial strains isolated from different organisms, it would be necessary to compare ColV derivatives from both human and animal sources.

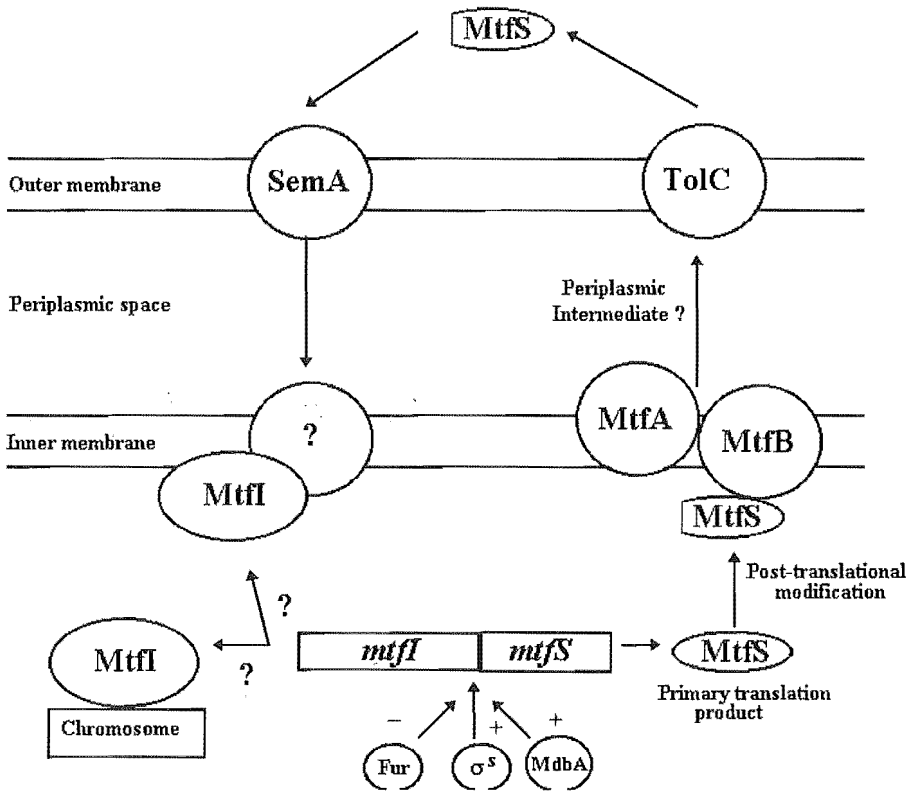


Figure 40. A suggested model for the biological activity of microcin 24. Negative regulation by the Fur repressor and positive regulation by σ^S and MdbA is denoted "-" and "+" respectively. Proteins or pathways not yet determined are denoted "?". Two possible mechanisms for immunity to Mcc24 are shown. In the first, MtfI prevents MtfS from entering the cell. The alternative is that MtfI prevents the nuclease activity of MtfS by binding to the cellular target of the toxin (DNA) or binding to the active site of MtfS.

4.5 CONCLUSION.

The study presented in this thesis represents the first analysis of Mcc24 and the genes involved in the production of this novel microcin. The identification of the genes involved in Mcc24 production, immunity, transport and regulation and their protein products, forms the basis for more in-depth analysis of the Mcc24 system and the interaction between the various components which make up this system. The exact mechanism by which a number of the proteins involved in Mcc24 production and activity function, although suggested, awaits further elucidation.

Four general areas of research into the molecular biology of Mcc24 require further investigation. The first area of study involves the mechanisms by which MdbA regulates the expression of Mcc24, in order to determine whether MdbA functions in a similar manner to H-NS. It would also be of interest to investigate whether MdbA mediated regulation was involved in the σ^S-dependent expression of Mcc24. Any investigation into the regulation of Mcc24 expression would also require the experimental confirmation of the putative promoter sequences identified by DNA sequencing in order to determine whether growth-phase dependent expression of Mcc24 was mediated by σ^S or σ^{70}, and to identify structural motifs involved in the regulation.

The specific role of the leader peptide in the production of Mcc24 is another area for future investigation. Whether the leader peptide of pre-MtfS has a role in regulating the activity of Mcc24 within the producing cell has yet to be fully determined. Also the mechanisms of post-translational modification and the role of the ABC transport complex comprising MtfA, MtfB and TolC in this process awaits further elucidation. Exploring the differences between the pre-MtfS and pre-ColV export signals in order to help understand the specific nature of the export signals and how they influence transport using MtfAB and CvaAB would also be of interest.

The third area of investigation would be to elucidate the exact mechanism by which Mcc24 induces DNA degradation in sensitive cells. Associated with this would be analysis into how Mtfl mediates the specific immunity to Mcc24.

Defining the role of Mcc24 in the virulence of *E. coli* forms the last area of interest. It would first be necessary to determine what other virulence factors are located on the plasmid p24-2. An assessment of the embryo lethality assay using ColV

plasmids derived from *E. coli* of human and avian origins would be of interest in order to investigate the appropriateness of this system in determining the toxicity of a microcin. Further investigations into the physical properties of Mcc24 are also required to determine whether this toxin remains active within the intestine or bladder.

BIBLIOGRAPHY.

Achtman, M., N. Kennedy, R. Skurray. 1977. Cell-cell interactions in conjugating *Escherichia coli*: Role of TraT protein in surface exclusion. Proc. Natl. Acad. Sci. USA. **74:**5104-5108.

Achtman, M., A. Mercer, B. Kusecek, A. Pohl, M. Heyzenroeder, W. Aaronson, A. Sutton, and R.P. Silver. 1983. Six widespread bacterial clones among *Escherichia coli* K1 isolates. Infect. Immun. **39:**315-335.

Adler, H.I., W.D. Fisher, A. Cohen, and A.A. Hardigree. 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. USA. **57:**321-326.

Aguilar, A., F. Baquero, J.C. Martínez, and C. Asensio. 1983. Microcin 15n: A second antibiotic from *Escherichia coli* LP15. J. Antibiot. **36:**325-327.

Aguilar, A., J.C. Pérez-Díaz, F. Baquero, and C. Asensio. 1982. Microcin 15m from *Escherichia coli*: Mechanism of antibiotic action. Antimicrob. Agents Chemo. **21:**381-386.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment tool. J. Mol. Biol. **215:**403-410.

Asensio, C., J.C. Pérez-Díaz, M.C. Martínez, and F. Baquero. 1976. A new family of low molecular weight antibiotics from enterobacteria. Biochem. Biophys. Res. Comm. **69:**7-14.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Strunl. 1989. Short protocols in molecular biology. Green Publishing Associates and Wiley-Interscience. New York, USA.

Aviv, M., H. Giladi, G. Schreiber, A.B. Oppenheim, and G. Glaser. 1994. Expression of the genes coding for the *Escherichia coli* integration host factor are controlled by growth phase, *rpoS*, ppGpp and by autoregulation. Mol. Microbiol. **14:**1021-1031.

Bagg, A., and J.B. Neilands. 1987. Molecular mechanisms of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51:**509-518.

Baquero, F., D. Bouanchaud, M.C. Martinez-Perez, and C. Fernandez. 1978. Microcin plasmids: a group of extrachromosomal elements coding for low -molecular- weight antiobiotics in *Escherichia coli*. J. Bacteriol. **135:**342-347.

Baquero, M.R., M. Bouzon, J. Varea, F. Moreno. 1995. *sbmC*, a stationary-phase induced SOS *Escherichia coli* gene, whose product protects cells from the DNA replication inhibitor microcin B17. Mol. Microbiol. **18:**301-311.

Baquero, F., and F. Moreno. 1984. The microcins. FEMS Microbiol. Lett. **23:**117-124.

Barth, M., C. Marschall, A. Muffler, D. Fischer, and R. Hengge-Aronis. 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of σ^S and many σ^S-dependent genes in *Escherichia coli*. J. Bacteriol. **177**:3455-3464.

Basyuk, E.I., B.M. Bezrukov, V.A. Lipasova, and I.A. Khmel'. 1994. Cloning and analysis of genetic determinants for microcins B2 and B27 production. Genetika. **30**:731-739.

Bayer, A., S. Freund, G. Nicholson, and G. Jung. (1993) Post-translational backbone modifications in the ribosomal biosynthesis of the glycine-rich antibiotic microcin B17. Angew. Chem. Int. Ed. Engl. **32**:1336-1339.

Bindereif, A., and J.B. Neilands. 1985. Aerobactin genes in clinical isolates of *Escherichia coli*. J. Bacteriol. **161**:727-735.

Binet, R., and C. Wandersman. 1995. Protein secretion by hybrid bacterial ABC-transporters: specific functions in the membrane ATPase and the membrane fusion protein. EMBO J. **14**:2298-2306.

Binns, M.M., D.L. Davies, and K.G. Hardy. 1979. Cloned fragments of the plasmid ColV,I-K94 specifying virulence and serum resistance. Nature **279**:778-781.

Binns, M.M., J. Mayden, and R.P. Levine. 1982. Further characterization of complement resistance conferred on *Escherichia coli* by the plasmid genes *traT* of R100 and *iss* of ColV, I-K94. Infect. Immun. **35**:654-659.

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513-1523.

Bohannon, D.E., N. Connell, J. Keener, A. Tormo, M. Espinosa-Urgel, M.M. Zambrano, and R. Kolter. 1991. Stationary-phase-inducible "gearbox" promoters: Differential effects of *katF* mutations and role of σ^{70}. J. Bacteriol. **173**:4482-4492.

Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heynecker, H.W. Boyer, J.H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene. **2**:95-113.

Bradley, D.E., and S.P. Howard. 1992. A new colicin that adsorbs to outermembrane protein Tsx but is dependent on the *tonB* instead of the *tolQ* membrane transport systems. J. Gen. Microbiol. **138**:2721-2724.

Braun, V., H. Pilsl, and P. Groβ. 1994. Colicins: Structures, modes of action, transfer through membranes, and evolution. Arch. Microbiol. **161**:199-206.

Brennan, R.G., and B.W. Mathews. 1989. The helix-turn-helix DNA binding motif. J. Biol. Chem. **264**: 1903-1906.

Calderwood, S.B., and J.J. Mekalanos. 1987. Iron regulation of shiga-like toxin expressed in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759-4764.

Calderwood, S.B., and J.J. Mekalanos. 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:**1015-1017.

Casadaban, M.J. 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:**541-555.

Cavard, D., and B. Oudega. 1992. General introduction to the secretion of bacteriocins, pp. 297-305. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol. 65. Springer-Verlag, Berlin.

Chang, A.C.Y., and S.N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. J. Bacteriol. **134:**1141-1156.

Chehade, H., and V. Braun. 1988. Iron-regulated synthesis and uptake of colicin V. FEMS Microbiol. Lett. **52:**177-182.

Chen, E.Y., and P.H. Seeburg. 1985. Supercoiling sequencing: A fast and simple method for sequencing plasmid DNA. DNA. **4:**165-170.

Clancey, J., and D.C. Savage. 1981. Another colicin V phenotype: *In vitro* adhesion of *Escherichia coli* to mouse epithelium. Infect. Immun. **32:**343-352.

Clarke-Curtiss, J.E., and R. Curtis III. 1983. Analysis of recombinant DNA using *Escherichia coli* minicells. Meth. Enzymol. **101:**347-362.

Cole, S.T., B. Saint-Joanis, A.P. Pugsley. 1985. Molecular characterization of the colicin E2 operon and identification of its products. Mol. Gen. Genet. **198:**465-472.

Colonna, B., M. Casalino, P.A. Fradiani, C. Zagaglia, S. Naitaz, L. Leoni, G. Prosseda, A. Coppo, P. Ghelardini, and M. Nicoletti. 1995. H-NS regulation of virulence gene expression in enteroinvasive *Escherichia coli* harbouring the virulence plasmid integrated into the chromosome. J. Bacteriol. **177:**4703-4712.

Compan, I., and D. Touati. 1993. Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. J. Bacteriol. **175:**1687-1696.

Cornelis, G.R., C. Sluiters, I. Delor, D. Geib, K. Kaniga, C. Lambert de Rouvroit, M.-P. Sory, J.-C. Vanooteghem, and T. Michiels. 1991. *ymoA*, a *Yersinia enterocolitica* chromosomal gene modulating the expression of virulence functions. Mol. Microbiol. **5:**1023-1034.

Crothers, D.M., T.E. Haran, and J.G. Nadeau. 1990. Intrinsically bent DNA. J. Biol. Chem. **13:**7093-7096.

Darken,J., and D.C. Savage. 1987. Influences of conjugal transfer functions of colicin V plasmids on adhesion of *Escherichia coli* to murine intestinal tissue. Infect. Immun. **55:**2483-2489.

Davagnino, J., M. Herrero, D. Furlong, F. Moreno, and R. Kolter. 1986. The DNA replication inhibitor microcin B17 is a forty-three-amino acid protein containing sixty percent gycine. Proteins. **1:**230-238.

Davies, J. 1990. What are antibiotics? Archaic functions for modern activities. Mol. Microbiol. **4:**1227-1232.

Davies, D.L., F.R. Falkiner, and K.G. Hardy. 1981. Colicin V production by clinical isolates of *Escherichia coli*. Infect. Immun. **31:**574-579.

de Graaf, F.K., and B. Oudega. 1986. Production and release of Cloacin DF13 and related colicins. Curr. Top. Microbiol. Immun. **125:**183-205.

del Castillo, I., J.M. Gómez, and F. Moreno. 1990. *mprA*, an *Escherichia coli* gene that reduces growth-phase-dependent synthesis of microcins B17 and C7 and blocks osmoinduction of *proU* when cloned on a high-copy-number plasmid. J. Bacteriol. **172:**437-445.

Delepelaire, P., and C. Wandersman. 1991. Protein secretion in gram-negative bacteria. J. Biol. Chem. **265:**17118-17125.

de Lorenzo, V. 1984. Isolation and characterization of microcin E492 from *Klebsiella pneumoniae*. Arch. Microbiol. **139:**72-75.

de Lorenzo, V., and A. Aguilar. 1984. Antibiotics from gram-negative bacteria: do they play a role in microbial ecology? TIBS. **9:**266-269.

de Lorenzo, V., J.L. Martínez, and C. Asensio. 1984. Microcin-mediated interactions between *Klebsiella pneumoniae* and *Escherichia coli* strains. J. Gen. Microbiol. **130:**391-400.

de Lorenzo, V., and A.P. Pugsley. 1985. Microcin E492, a low-molecular weight peptide antibiotic which causes depolarization of the *Escherichia coli* cytoplasmic membrane. Antimicrob. Agents Chemo. **27:**666-669.

de Lorenzo, V., W. Sechan, M. Herrero, and J.B. Neilands. 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. J. Bacteriol. **169:**2624-2630.

Dersch, P., K. Schmidt, and E. Bremer. 1993. Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA binding protein H-NS is subject to growth phase control and autoregulation. Mol. Microbiol. **8:**875-889.

de Vos, W.M., O.P. Kuipers, J.R. van der Meer, and R.J. Siezen. 1995. Maturation pathway of nisin and other lantibiotics posttranslationally modified antimicrobial peptides exported by gram-positive bacteria. Mol. Microbiol. **17**:427-437.

Diaz-Guerra, L., F. Moreno, and J.L. San Millán. 1989. *appR* gene product activiates transcription of microcin C7 plasmid genes. J. Bacteriol. **171**:2906-2908.

Dinh, T., I.T. Paulsen, and M.H. Saier. 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176**:3825-3831.

Doige, C.A., and G.F.-L. Ames. 1993. ATP-dependent transport systems in bacteria and humans: Relevance to cystic fibrosis and multidrug resistance. Ann. Rev. Microbiol. **47**:291-319.

Dorman, C.J., N.N. Bhriain, and C.F. Higgins. 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri.* Nature. **344**:789-792.

Drlica, K., and J. Rouviere-Yaniv. 1987. Histone like proteins of bacteria. Microbiol. Rev. **51**:301-319.

Duro, A.F., R. Serrano, and C. Asensio. 1979. Effect of the antibiotic microcin 140 on the ATP level and amino acid transport of *Escherichia coli.* Biochem. Biophys. Res. Comm. **88**:297-304.

Endicott, J.A., and V. Ling. 1989. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. **58**:137-171.

Englard, S., and S. Seifter. 1990. Purification procedures: Precipitation techniques. Meth. Enzymol. **182**:285-306.

Espinosa-Urgel, M., and A. Torma. 1993. σ^S-dependent promoters in *Escherichia coli* are located in DNA regions with intrinsic curvature. Nucleic Acids Res. **21**:3667-3670.

Falconi, M., N.P. Higgins, R. Spurio, C.L. Pon, and C.O. Gualerzi. 1993. Expression of the gene encoding the major bacterial nucleoid protein H-NS is subject to transcriptional autorepression. Mol. Microbiol. **10**:273-282.

Fang, F.C., S.J. Libby, N.A. Buchmeier, P.C. Loewen, J. Switala, J. Harwood, and D.G. Guiney. 1992. The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA. **89**:11978-11982.

Fath, M.J., and R. Kolter. 1993. ABC transporters: Bacterial exporters. Microbiol. Rev. **57**:995-1017.

Fath, M.J., R. C. Skvirsky, L. Gilson, H.K. Mahanty, and R. Kolter. 1992. The secretion of colicin V, pp. 331-348. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol. 65. Springer-Verlag, Berlin.

Fath, M.J., R.C. Skvirsky, and R. Kolter. 1991. Functional complementation between bacterial MDR--like export systems: colicin V, alpha-hemolysin and *Erwinia* protease. J. Bacteriol. **173:**7549-7556.

Fath, M.J., L.-H. Zhang, J. Rush, and R. Kolter. 1994. Purification and characterization of colicin V from *Escherichia coli*. Biochemistry. **33:**6911-6917.

Fernandez-Beroz, M.E., V. Kissel, H. Lior, and F.C. Cabella. 1990. Virulence-related genes in ColV plasmids of *Escherichia coli* isolated from human blood and intestines. J. Clin. Microbiol. **28:**742-746.

Forsman, K., B. Sondén, M. Göransson, and B.E. Uhlin. 1992. Antirepression function *in Escherichia coli* for the cAMP-cAMP receptor protein transcriptional activator. Proc. Natl. Acad. Sci. USA. **89:**9880-9884.

Fredericq, P., E. Joiris, M. Betz-Barreau, and A. Gratia. 1949. Researche des germes proucteurs do colicins dans les selles de malades atteints de fievre paratyphoide B. C.R. Soc. Biol. **143:**556-559.

Free, A., and C.J. Dorman. 1995. Coupling of *Escherichia coli hns* mRNA levels to DNA synthesis by autoregulation: implications for growth phase control. Mol. Microbiol. **18:**101-113.

Freundlich, M., N. Ramani, E. Mthew, A. Sirko, and P. Tsui. 1992. The role of integration host factor in gene expression in *Escherichia coli*. Mol. Microbiol. **6:**2557-2563.

Frick, K.K., R.L. Quackenbush, and J. Konisky. 1981. Cloning of immunity and structural genes for colicin V. J. Bacteriol. **148:**498-507.

Friedman, D.I. 1988. Integration host factor: A protein for all reasons. Cell. **55:**545-554.

Gaggero, C., F. Moreno, and M. Laviña. 1993. Genetic analysis of microcin H47 antibiotic system. J. Bacteriol. **175:**5420-5427.

Garcia-Bustos, J.F., N. Pezzi, and C. Asensio. 1984. Microcin 7: Purification and properties. Biochem. Biophys. Res. Comm. **119:**779-785.

Garcia-Bustos, J.F., N. Pezzi, and E. Mendez. 1985. Structure and mode of action of microcin 7, an antimicrobial peptide produced by *Escherichia coli*. Antimicrob. Agents Chemo. **27:**791-797.

Garrido, M.C., M. Herrero, R. Kolter, and F. Moreno. 1988. The export of the DNA replication inhibitor microcin B17 provides immunity for the host cell. EMBO J. **7:**1853-1862.

Géli, V., and C. Lazdunski. 1992. Immunity protein to pore forming colicins, pp. 171-179. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol. 65. Springer-Verlag, Berlin.

Genilloud, O., F. Moreno, and R. Kolter. 1989. DNA sequence, products and transcription pattern of the genes involved in production of the DNA replication inhibitor microcin B17. J. Bacteriol. **171:**1126-1135.

Gentry, D.R., V.J. Hernandez, L.H. Hguyen, P.B. Jensen, and M. Cashel. 1993. Synthesis of the stationary-phase sigma factor σ^S is positively regulated by ppGpp. J. Bacteriol. **175:**7982-7989.

Gilson, L., H.K. Mahanty, and R. Kolter. 1987. Four plasmid genes are required for colicin V synthesis, export and immunity. J. Bacteriol. **169:**2466-2470.

Gilson, L., H.K. Mahanty, and R. Kolter. 1990. Genetic analysis of an MDR-like export system: the secretion of colicin V. EMBO J. **9:**3875-3884.

González-Pastor, J.E., J.L. San Millán, M.A. Castilla, and F. Moreno. 1995. Structure and organization of plasmid genes required to produce the translation inhibitor Microcin C7. J. Bacteriol. **177:**7131-7140.

González-Pastor, J.E., J.L. San Millán, and F. Moreno. 1994. The smallest known gene. Nature. **369:**281.

Goodrich, J.A., M.L. Schwartz, and W.R. McClure. 1990. Searching for and predicting the activity of sites for DNA binding proteins: compilation and analysis of the binding sites for *Escherichia coli* integration host factor (IHF). Nucleic Acids Res. **18:**4993-5000.

Goosen, N., and P. van de Puttle. 1995. The regulation of transcription initiation by integration host factor. Mol. Microbiol. **16:**1-7.

Grant, S.G.N., J. Jessee, F.R. Bloom, and D. Hanahan. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA. **87:**4645-4649.

Gratia, A. 1925. Sur un remarquable exemple d'antagonisime entr deux souches do Colibacille. C.R. Soc. Biol. **93:**1040-1041.

Guerinot, M.L. 1994. Microbial iron transport. Ann. Rev. Microbiol. **48:**743-772.

Guijarro, J.I., J.E. González-Pastor, F. Baleux, J.L. San Millán, M. Rico, F. Moreno, and M. Delepierre. 1995. Chemical structure and translation inhibition studies of the antibiotic microcin C7. J. Biol. Chem. **270:**23520-23532.

Guiney, D.G., F.C. Fang, M. Krause, S. Libby, N.A. Buchmeier, and J. Fierer. 1995. Biology and clinical significance of virulence plasmids in *Salmonella* serovars. Clin. Infect. Dis. **21:**S146-S151.

Guzzo, J., F. Duong, C. Wandersman, M. Murigier, and A. Lazdunski. 1991. The secretion genes of *Pseudomonas aeruginosa* alkaline protease are functionally related to those of *Erwinia chysanthemi* proteases and *Escherichia coli* α-hemolysin. Mol. Microbiol. **5**:447-453.

Hansen, J.N. 1993. Antibiotic synthesis by post-translational modification. Ann. Rev. Microbiol. **47**:535-564.

Hardy, K.G. 1975. Colicinogeny and related phenomena. Bacteriol. Rev. **39**:464-515.

Harkness, R.E., and T. Ölschläger. 1991. The biology of colicin M. FEMS Microbiol. Rev. **88**:27-42.

Harrington, R.E. 1992. DNA curving and bending in protein-DNA-recognition. Mol. Microbiol. **6**:2549-2555.

Håvarstein, L.S., D.B. Diep, and I.F. Ness. 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substances concomitant with export. Mol. Microbiol. **16**:229-240.

Håvarstein, L.S., H. Holo, and I.F. Nes. 1994. The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by gram-positive bacteria. Microbiol. **140**:2383-2389.

He, M., H. Liu, Y. Wang, and B. Austen. 1992. Optimized centrifugation for rapid elution of DNA from agarose gels. GATA. **9**:31-33.

Helmann, J.D., and M.J. Chamberlin. 1988. Structure and function of bacterial sigma factors. Ann. Rev. Biochem. **57**:839-872.

Hernández-Chico, C., M. Herrero, M. Rejas, J.L. San Millán, and F. Moreno. 1982. Gene *ompR* and regulation of microcin 17 and colicin E2 synthesis. J. Bacteriol. **152**:897-900.

Hernández-Chico, C., O. Mayo, J.L. Vizán, M. Laviña, and F. Moreno. 1992. Uptake and mode of action of the peptide antibiotic microcin B17, pp. 15-17. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol 65. Springer-Verlag, Berlin.

Hernández-Chico, C., J.L. San Millán, R. Kolter, and F. Moreno. 1986. Growth phase and OmpR regulation of transcription of Microcin B17 genes. J. Bacteriol. **167**:1058-1065.

Herrero, M., R. Kolter, and F. Moreno. 1986. Effects of Microcin B17 on Microcin B17-immune cells. J. Gen. Microbiol. **132**:403-410.

Herrero, M., and F. Moreno. 1986. Microcin B17 blocks DNA replication and induces the SOS system in *Escherichia coli.* J. Gen. Microbiol. **132**:393-402.

Higgins, C.F. 1992. ABC transporters: from microorganism to man. Ann. Rev. Cell Biol. **8**:67-113.

Higgins, C.F., C.J. Dorman, D.A. Stirling, L. Waddell, I.R. Booth, G. May, and E. Bremer. 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. Cell. **52**:569-584.

Higgins, C.F., I.D. Hiles, G.P.C. Salmond, D.R. Gill, J.A. Downie, I.J. Evans, I.B. Holland, C. Grey, S.D. Buckel, A.W. Bell, and M.A. Hermodson. 1986. A family of related ATP-binding units coupled to many distinct biological processes in bacteria. Nature **332**:448-450.

Higgins, C.F., J.C.D. Hinton, C.S.J. Hulton, T. Owen-Hughs, G.D. Pavitt, and A. Seirafi. 1990. Protein H1: a role for chomatin structure in the regulation of bacterial gene expression and virulence? Mol. Microbiol. **4**:2007-2012.

Hopwood, D.A. 1978. Extrachromosomally determined antibiotic production. Ann. Rev. Microbiol. **32**:373-392.

Hulton, C.S.J., A. Seirafi, J.C.D. Hinton, J.M. Sidebotham, L. Waddell, G.D. Pavitt, T. Owen-Hughs, A. Spassky, H. Buc, and C.F. Higgins. 1990. Histone-like protein H1 (H-NS), DNA supercoiling and gene expression in bacteria. Cell. **63**:631-642.

Hyde, S.C., P. Emsley, M.J. Hartshorn, M.M. Mimmack, U. Gileadi, S.R. Pearce, M.P. Gallagher, D.R. Gill, R.E. Hubbard, and C.F. Higgins. 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature **346**:362-365.

Iida, S., J. Meyer, and W. Arber. 1983. Prokaryotic IS elements, pp. 159-221. **In** J.A. Shapiro (ed.), Mobile genetic elements. Academic Press Inc., Orlando, Florida.

Ish-Horowicz, D., and J.F. Burke. 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989-2998.

Jack, R.W., J.R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59**:171-200.

Jakes, K.S., and C. Lazdunski. 1992. Immunity to colicins, pp. 163-170. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol. 65. Springer-Verlag, Berlin.

Jishage, M., and A. Ishihama. 1995. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: Intracellular, levels of σ^{70} and σ^{38}. J. Bacteriol. **177**:6832-6835.

Kadner, R.J. 1990. Vitamin B12 transport in *Escherichia coli*: energy coupling between membranes. Mol. Microbiol. **4**:2027-2033.

Kennedy, M.A. 1988. Molecular genetics protocols. Cytogenetics and Molecular Oncology Unit. Christchurch Hospital.

Kenny, B., S. Taylor, and I.B. Holland. 1992. Identification of individual amino acids required for secretion within the hemolysin (HylA) C-terminal targeting region. Mol. Microbiol. **6:**1477-1489.

Khmel, I.A., V.M. Bondarenko, I.M. Manokhina, E.I. BAsyuk, A.Z. Metlitskaya, V.A. Lipasova, and Y.M. Romanova. 1993. Isolation and characterization of *Escherichia coli* strains producing microcins of B and C types. FEMS Microbiol. Lett. **111:**269-274.

Kippenberger, A.G. 1996. Further characterisation of the genetic determinants of microcin J, and an investigation into microcins and colicins as virulence factors. Master of Science, Thesis. University of Canterbury, Christchurch.

Kleckner, N., J. Bender, and S. Gottesman. 1991. Uses of transposons with emphasis on Tn*10*. Meth. Enzymol. **204:**139-180.

Kleinkauf, H., and H. von Döhren. 1987. Biosynthesis of peptide antibiotics. Ann. Rev. Microbiol. **41:**259-289.

Kolter, R., and F. Moreno. 1992. Genetics of ribosomally synthesized peptide antibiotics. Ann. Rev. Microbiol. **46:**141-163.

Kolter, R., D.A. Seigele, and A. Torma. 1993. The stationary phase of the bacterial life cycle. Ann. Rev. Microbiol. **47:**855-874.

Konisky, J. 1982. Colicins and other bacteriocins with established modes of action. Ann. Rev. Microbiol. **36:**125-144.

Koronakis, E., C. Hughs, I. Milisav, and V. Koronakis. 1995. Protein exporter function and *in vitro* ATPase activity are correlated in ABC-domain mutants of HylB. Mol. Microbiol. **16:**87-96.

Kowarz, L., C. Coynault, V. Robbe-Saule, and F. Norel. 1994. The *Salmonella typhimurium katF* (*rpoS*) gene: Cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. J. Bacteriol. **176:**6852-6860.

Kurepina, N.E., A.Z. Metlitskaia, and I.A. Khmel'. 1992. Mapping of plasmid genes responsible for microcin R51 synthesis and immunity to it. Genetika. **28:**17-24.

Kurepina, N.E., E.I. Basyuk, A.Z. Metlitskaya, D.A. Zaitsev, and I.A. Khmel. 1993. Cloning and mapping of the genetic determinants for Microcin C51 production and immunity. Mol. Gen. Genet. **241:**700-706.

Kurepina, N.E., I.A. Khmel, V.A. Lipasova, and D.A. Zaitsev. 1990. Microcin R51 and a plasmid determining its synthesis. Molek. Genetik. Mikrobiol, Vir. **5:**12-17.

Kyte, J., and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105-132.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

Lagos, R., M. Wilkens, C. Vergara, X. Cecchi, and O. Monasterio. 1993. Microcin E492 forms ion channels in phospholipid bilayer membranes. FEBS Lett. **321**:145-148.

Lakey, J.H., J.M. González-Mañas, F.G. van der Goot, and F. Pattus. 1992. The membrane insertion of colicins. FEBS Lett. **307**:26-29.

Lakey, J.H., F.G. van der Goot, and F. Pattus. 1994. All in the family: the toxic activity of pore-forming colicins. Toxicology. **87**:85-108.

Lange, R., M. Barth, and R. Hengge-Aronis. 1993. Complex transcriptional control of the σ^S-dependent stationary-phase-induced and osmotically regulated *osmY* (*csi-5*) gene suggests novel roles for Lrp, cyclic AMP (cAMP) receptor protein-cAMP complex and integration host factor in the stationary phase. J. Bacteriol. **175**:7910-7917.

Lange, R., D. Fischer, and R. Hengge-Aronis. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the σ^S subunit of RNA polymerase in *Escherichia coli*. J. Bacteriol. **177**:4674-4680.

Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5**:49-59.

Lange, R., and R. Hengge-Aronis. 1994. The cellular concentration of σ^S subunit of RNA polymerase in *Escherichia coli* is controlled at the level of transcription, translation and protein stability. Genes Develop. **8**:1600-1612.

La Teana, A., A. Brandi, M. Falconi, R. Spurio, C.L. Pon, and C.O. Gualerzi. 1991. Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleiod protein H-NS. Proc. Natl. Acad. Sci. USA. **88**:10907-10911.

Laviña, M., and C. Gaggero. 1992. Genetic determinants for microcin H47, an *Escherichia coli* chromosome-encoded antibiotic, pp. 413-415. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol. 65. Springer-Verlag, Berlin.

Laviña, M., C. Gaggero, and F. Moreno. 1990. Microcin H47, a chromosome-encoded microcin antibiotic of *Escherichia coli*. J. Bacteriol. **172**:6585-6588.

Laviña, M., A.P. Pugsley, and F. Moreno. 1986. Identification, mapping, cloning and characterisation of a gene (*sbmA*) required for microcin B17 action on *Escherichia coli* K-12. J. Gen. Microbiol. **132**:1685-1693.

Lazdunski, C.J., D. Baty, V. Géli, D. Cavard, J. Morlon, R. Lloubés, S.P. Howard, M. Knibiehler, M. Chartier, S. Varenne, M. Frenette, J.-L. Dasseux, and F. Pattus. 1988. The membrane channel-forming colicin A: synthesis, secretion, activity and immunity. Biochim. Biophys. Acta. **947**:445-464.

Le Cam, E., D. Frechon, M. Burray, A. Fourcade, and E. Delain. 1994. Observation of binding and polymerization of the Fur repressor onto operator-containing DNA with electron and atomic force microscopes. Proc. Natl. Acad. Sci. USA. **91**:11816-11820.

Létoffé, S., P. Delepelaire, and C. Wandersman. 1990. Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions are analogous to those for *Escherichia coli* α-hemolysin. EMBO J. **9**:1375-1382.

Liu, J. 1994. Microcin B17: Posttranslational modifications and their biological implications. Proc. Natl. Acad. Sci. USA. **91**:4618-4620.

Litwin, C.M., and S.B. Calderwood. 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6**:137-149.

Loewen, P.C., and R. Hengge-Aronis. 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. Ann. Rev. Microbiol. **48**:53-80.

Loewen, P.C., I. von Ossowski, J. Switala, and M.R. Mulvey. 1993. KatF (σ^S) synthesis in *Escherichia coli* is subject to posttranscriptional regulation. J. Bacteriol. **175**:2150-2153.

Lonetto, M., M. Gribskov, and C.A. Gross. 1992. The σ^{70} family: sequence conservation and evolutionary relationships. J. Bacteriol. **174**:3843-3849.

Lopilato, J., S. Bortner, and J. Beckwith. 1986. Mutations in a new chromosomal gene *of Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. Mol. Gen. Genet. **205**:285-290.

Luria, S.E., and J.L. Suit. 1986. Colicins and Col plasmids, pp. 1615-1648. *In* F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, and H.E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. Vol. 2. American Society for Microbiology, Washington DC.

Martin, J.E. 1994. Characterization of the genetic determinants encoding microcin J, a novel microcin. Master of Science, Thesis. University of Canterbury, Christchurch.

Martin, J.F., and A.L. Demain. 1980. Control of antibiotic biosynthesis. Microbiol. Rev. **44**:230-251.

Martínez, J.L., and J.C. Pérez-Díaz. 1986. Isolation, characterization, and mode of action on *Escherichia coli* strains of microcin D93. Antimicrob. Agents Chemo. **29**:456-460.

Martínez, J.L., and J.C. Pérez-Díaz. 1990. Cloning of the determinants for microcin D93 production and analysis of three different D-type microcin plasmids. Plasmid. **23:**216-225.

Mayo, O.C., C. Hernández-Chico, and F. Moreno. 1988. Microcin B17, a novel tool for preparation of maxicells: Identification of polypeptides encoded by the IncFII minireplicon pMccB17. J. Bacteriol. **170:**2414-2417.

Mayr-Harting, A., A.J. Hedges, and R.C.W. Berkeley. 1972. Methods for studying bacteriocins, pp. 315-422. *In* J.R. Norris, and D.W. Ribbons (ed.), Methods in Microbiology. 7A. Academic Press, London.

McCann, M.P., C.D. Fraley, and A. Matin. 1993. The putative σ factor KatF is regulated posttranscriptionally during carbon starvation. J. Bacteriol. **175:**2143-2149.

McCann, M.P., J.P. Kidwell, and A. Matin. 1991. The putative σ factor KatF has a central role in developement of starvation-mediated resistance in *Escherichia coli*. J. Bacteriol. **173:**4188-4194.

Meagher, R.B., R.C. Tait, M. Betlach, and H.W. Boyer. 1977. Protein expression in *E. coli* by recombinant plasmids. Cell. **10:**521-536.

Messing, J., B. Gronenborn, B. Muller-Hill, and P.H. Hofschneider. 1977. Filamentous coliphage M13 as a cloning vehicle: Insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*. Proc. Natl. Acad. Sci. USA. **74:**3642-3646.

Metlitskaya, A.Z., G.S. Katrukha, A.S. Shashkov, D.A. Zaitsev, TsA. Egorov, and I.A. Khmel. 1995. Structure of microcin C51, a new antibiotic with a broad spectrum of activity. FEBS Lett. **357:**235-238.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

Moll, A., P.A. Manning, and K.N. Timmis. 1980. Plasmid-determined resistence to serum bacteriocidal activity: a major outer membrane protein, the *traT* gene product, is responsible for plasmid-specified serum resistence in *Escherichia coli*. Infect. Immun. **28:**359-367.

Montenegro, M.A., D. Bitter-Suermann, J.K. Timmis, M.E. Aguero, F.C. Cabello, S.C. Sanyal, and K.N. Timmis. 1985. *traT* gene sequences, serum resistance and pathogenicity-related factors in clinical isolates of *Escherichia coli* and other gram-negative bacteria. J. Gen. Microbiol. **131:**1511-1521.

Moreno, F., J.L. San Millán, I. del Castillo, J.M. Gómez, M.C. Rodríguez-Sáinz, J.E. González-Pastor, and L. Díaz-Guerra. 1992. *Escherichia coli* genes regulating the production of microcins MccB17 and MccC7, pp. 3-13. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol65. Springer-Verlag, Berlin.

Muffler, A., D. Fischer, S. Altuvia, G. Storz, and R. Hengge-Aronis. 1996. The response regulator RssB contols stability of the σ^S subunit of RNA polymerase in *Escherichia coli*. EMBO J. **15:**1333-1339.

Mulvey, M.R., and P.C. Loewen. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests *katF* protein is a novel σ transcription factor. Nucleic Acids Res. **17:**9979-9991.

Murray, N.E., W.J. Brammar, and K. Murray. 1977. Lambdoid phages that simplify the recovery of in vitro recombinations. Mol. Gen. Genet. **150:**53-61.

Nguyen, L.H., D.B. Jensen, N.E. Thompson, D.R. Gentry, and R.R. Burgess. 1993. *In vitro* functional characterization of overproduced *Escherichia coli katF/ropS* gene product. Biochemistry. **32:**11112-11117.

Nilius, A.M., and D.C. Savage. 1984. Serum resistance encoded by colicin V plasmids in *Escherichia coli* and its relationship to the plasmid transfer system. Infect. Immun. **43:**947-953.

Novoa, M.A., L. Díaz-Guerra, J.L. San Millán, and F. Moreno. 1986. Cloning and mapping of the genetic determinants for microcin C7 production and immunity. J. Bacteriol. **168:**1384-1391.

O'Brien, G.J. 1992. The association between colicinogenicity and pathogenesis among clinical isolates of *Escherichia coli* causing urinary tract infections: The identification of a new colicin, colicin 24. Master of Science, Thesis. University of Canterbury, Christchurch.

O'Brien, G.J., S.T. Chambers, B. Peddie, and H.K. Mahanty. 1996. The association between colicinogenicity and pathogenicity among uropathogenic isolates of *Escherichia coli*. Microb. Path. **20:**185-190.

O'Brien, G.J., and H.K. Mahanty. 1994. Colicin 24, a new plasmid-borne colicin from a uropathogenic strain of *Escherichia coli*. Plasmid. **31:**288-296.

Ochsner, U.A., A.I. Vasil, and M.L. Vasil. 1995. Role of the ferric uptake regulator *of Pseudomonas aeruginosa* in the regulation of siderophores and Exotoxin A expression: Purification and activity on iron-regulated promoters. J. Bacteriol. **177:**7194-7201.

Ölschläger, T. 1991. A colicin M derivative containing the lipoprotein signal sequence is secreted and renders the colicin M target accessible from inside the cells. Arch. Microbiol. **156:**449-454.

Owen-Hughes, T.A., G.D. Pavitt, D.S. Santos, J.M. Sidebotham, C.S.J. Hulton, J.C.D. Hinton, and C.F. Higgins. 1992. The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell. **71:**255-265.

Pabo, C.O., and R.T. Sauer. 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53:**293-321.

Palmer, L.R., and A.R. Merril. 1994. Mapping the membrane topology of the closed state of the colicin E1 channel. J. Biol. Chem. **269:**4187-4193.

Pattus, F., D. Massotte, H.U. Wilmsen, J. Lakey, D. Tsernoglou, A. Tucker, and M.W. Parker. 1990. Colicins: Prokaryotic killer-pores. Experientia. **46:**180-192.

Pearce, S.R., M.L. Mimmack, M.P. Gallagher, U. Gileadi, S.C. Hyde, and C.F. Higgins. 1992. Membrane topology of the intergral membrane components OppB and OppC, of the oligopeptide permease of *Salmonella typhimurium*. Mol. Microbiol. **6:**47-57.

Pearson, W.R., and D.J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. **85:**2444-2448.

Pérez-Díaz, J.C., and R.C. Clowes. 1980. Physical characterization of plasmids determining synthesis of a microcin which inhibits methionine synthesis in *Escherichia coli*. J. Bacteriol. **141:**1015-1023.

Pérez-Martín, J., F. Rojo, and V. de Lorenzo. 1994. Promoters responsive to DNA bending: a common theme in prokaryotic gene expression. Microbiol. Rev. **58:**268-290.

Pharmacia Fine Chemicals. 1979. Gel filtration theory and practice. 0Rahms i Lund. Sweden.

Pilsl, H., and V. Braun. 1995. Novel colicin 10: assignment of four domains to TonB- and TolC-dependent uptake via the Tsx receptor and to pore formation. Mol. Microbiol. **16:**57-67.

Pon, C.L., R.A. Calogero, and C.O. Gualerzi. 1988. Identification, cloning, nucleotide sequence and chromosomal map location of *hns*, the structural gene for *Escherichia coli* DNA-binding protein H-NS. Mol. Gen. Genet. **212:**199-202.

Postle, K. 1990. TonB and the gram-negative dilemma. Mol. Microbiol. **4:**2019-2025.

Pritchard, R.H., and I.B. Holland. 1985. Basic cloning techniques: A manual of experimental procedures. Blackwell Scientific Publications. London.

Pugsley, A.P. 1984a. The ins and outs of colicins. Part 1: Production and translocation across membranes. Microbiol. Sci. **1:**168-175.

Pugsley, A.P. 1984b. The ins and outs of colicins. Part II: Lethal action, immunity and ecological implications. Microbiol. Sci. **1:**203-205.

Pugsley, A.P. 1985. *Escherichia coli* K-12 strains for use for identification and characterization of colicins. J. Gen. Microbiol. **131:**369-376.

Pugsley, A.P., F.P. Moreno, and V. de Lorenzo. 1986. Microcin-E492-insensitive mutants *of Escherichia coli* K-12. J. Gen. Microbiol. **132:**3253-3259.

Pugsley, A.P., and B. Oudega. 1987. Methods for studying colicins and their plasmids, pp. 105-161. *In* K.G. Hardy (ed.), Plasmids, a practical approach. IRL Press, Oxford.

Quackenbush, R.L., and S. Falkow. 1979. Relationship between colicin V activity and virulence in *Escherichia coli*. Infect. Immun. **24:**562-564.

Reid, G., and J.D. Sorbel. 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Rev. Infect. Dis. **9:**470-487.

Riley, M.A., and D.M. Gordon. 1992. A survey of Col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of Col-plasmid lineages. J. Gen. Microbiol. **138:**1345-1352.

Rimsky, S., and A. Spassky. 1990. Sequence determinants for HI binding on *Escherichia coli lac* and *gal* promoters. Biochemistry. **29:**3765-3771.

Riordan, J.R., J.M. Rommens, B.-S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, M.L. Drumm, M.C. Iannuzzi, F.S. Collins, and L.-C. Tsui. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. **245:**1066-1072.

Rodriguez, R.L., and R.C. Tait. 1983. Recombinant DNA techniques: An introduction. Addison-Wesley Publishing Company. Massachusettes, USA.

Rodriguez-Sáinz, M.C., C. Hernández-Chico, and F. Moreno. 1990. Molecular characterization of *pmbA*, an *Escherichia coli* chromosomal gene required for the production of an antibiotic peptide MccB17. Mol. Microbiol. **4:**1921-1932.

Ruhr, E., and H.-G. Sahl. 1985. Mode of action of the peptide antibiotic Nisin and influences on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemo. **27:**841-845.

Sahl, H.-G. 1992. Introduction to the lantibiotics session, pp. 71-73. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol 65. Springer-Verlag, Berlin.

Sahl, H.-G. 1994. Gene encoded antibiotics made in bacteria, pp. 27-51. *In*, Antimicrobial peptides. Ciba Foundation Symposium 186. Wiley, Chichester.

Sahl, H.-G., and H. Brandis. 1983. Efflux of low-Mr substances from the cytoplasm of sensitive cells caused by the staphylococcin-like agent Pep5. FEMS Microbiol. Lett. **16:**75-79.

Saito, T., D. Daly, and R.J.P. Williams. 1991. The histidines of the iron-uptake regulation protein. Eur. J. Biochem. **197:**39-42.

Salomón, R.A., and R.N. Farías. 1992. Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. J. Bacteriol. **174:**7428-7435.

Salomón, R.A., and R.N. Farías. 1993. The FhuA protein is involved in microcin 25 uptake. J. Bacteriol. **175:**7741-7742.

Salomón, R.A., and R.N. Farías. 1994. Influence of iron on microcin 25 production. FEMS Microbiol. Lett. **121:**275-280.

Salomón, R.A., and R.N. Farías. 1995. The peptide antibiotic microcin 25 is imported through the TonB pathway and the SbmA protein. J. Bacteriol. **177:**3323-3325.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd ed. Vol. 3. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Sancar, A., A.M. Hack, and W.D. Rupp. 1979. Simple method for identification of plasmid-encoded proteins. J. Bacteriol. **137:**692-693.

Sanger, F., S. Hicklen, and A.R. Coulsen. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. **74:**5463-5467.

San Millán, J.L., C. Hernandez-Chico, P. Pereda, and F. Moreno. 1985a. Cloning and mapping of the genetic determinants for microcin B17 production and immunity. J. Bacteriol. **163:**272-281.

San Millán, J.L., R. Kolter, and F. Moreno. 1985b. Plasmid genes required for microcin B17 production. J. Bacteriol. **163:**1016-1020.

Schmid, M.B. 1990. More than just 'histone-like' proteins. Cell. **63:**451-453.

Schüller, F., R. Benz, and H.-G. Sahl. 1989. The peptide antibiotic subtilin acts by formation of voltage-dependent multi-state pores in bacterial and artificial membranes. Eur. J. Biochem. **182:**181-186.

Schweder, T., K.-H. Lee, O. Lomovskaya, and A. Matin. 1996. Regulation of *Escherichia coli* starvation phase sigma factor (σ^S) by ClpXP protease. J. Bacteriol. **178:**470-476.

Short, J.M., J.M. Fernandez, J.A. Sorge, and W.D. Huse. 1988. λZAP: A bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Res. **16:**7583-7600.

Shum, T.S. 1992. Molecular analysis of a chitinase locus in *Serratia entomophila*. Master of Science, Thesis. University of Canterbury, Christchurch.

Siegele, D.A., and R. Kolter. 1992. Life after Log. J. Bacteriol. **174:**345-348.

Silver, S., and M. Walderhaug. 1992. Gene regulation of plasmid- and chromosomal-determined inorganic ion transport in bacteria. Microbiol. Rev. **56:**195-228.

Skvirsky, R.C., S. Reginald, and X. Shen. 1995. Topology analysis of colicin V export protein CvaA in *Escherichia coli.* J. Bacteriol. **177:**6153-6159.

Smith, H.W. 1974. A search for transmissible pathogenic characters in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated, or identical with colicine V. J. Gen. Microbiol. **83:**95-111.

Smith, H.W., and M.B. Huggins. 1976. Further observations on the association of the colicin V plasmid of *Escherichia coli* with pathogenicity and survival in the alimentary tract. J. Gen. Microbiol. **92:**335-350.

Spassky, A., S. Rimsky, H. Garreau, and H. Buc. 1984. H1a, an *E. coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro.* Nucleic Acids Res. **12:**5321-5340.

Tagg, J.R. 1992. Bacteriocins of gram-positive bacteria: an opinion regarding their nature, nomenclature and numbers, pp. 33-35. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocin, Microcins and Lantibiotics. NATO ASI Series H: Cell Biology, Vol. 65. Springer-Verlag, Berlin.

Tagg, J.R., A.S. Dajani, and L.W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40:**722-756.

Takayanagi, Y., K. Tanaka, and H. Takahashi. 1994. Structure of the 5' upstream region and the regulation of the *rpoS* gene of *Escherichia coli.* Mol. Gen. Genet. **243:**525-531.

Tanaka, K., S. Kusano, N. Fujita, A. Ishihama, and H. Takahashi. 1995. Promoter determinants for *Escherichia coli* RNA polymerase holenzyme containing σ^{38} (the *rpoS* gene product). Nucleic Acids Res. **23:**827-834.

Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi. 1993. Heterogeneity of the principle sigma factor in *Escherichia coli*: The *rpoS* gene product, σ^{38}, is a second principle σ factor of RNA polymerase in stationary-phase *Escherichia coli.* Proc. Natl. Acad. Sci. USA. **90:**3511-3515.

Thomas, J.O., and R.D. Kornberg. 1978. The study of histone-histone associations by chemical cross-linking, pp. 429-440. *In* S. Stein, J. Stein, and L.J. Kleinsmith (ed.), Methods in cell biology. Academic Press, New York, .

Tippner, D., H. Afflerbach, C. Bradaczek, and R. Wagner. 1994. Evidence for a regulatory function of the histone-like *Escherichia coli* protein H-NS in ribosomal RNA synthesis. Mol. Microbiol. **11:**589-604.

Toba, M., H. Masaki, and T. Ohta. 1988. Colicin E8, a DNase which indicates an evolutionary relationship between colicins E2 and E3. J. Bacteriol. **170:**3237-3242.

Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa. 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: Activation by VirF and repression by H-NS. J. Bacteriol. **175:**6142-6149.

Travers, A., and A. Klug. 1987. DNA wrapping and writhing. Nature. **327:**280-281.

Ueguchi, C., M. Kakeda, and T. Mizuno. 1993. Autoregulatory expression of the *Escherichia coli hns* gene encoding a nucleoid protein: H-NS functions as a repressor of its own transcription. Mol. Gen. Genet. **236:**171-178.

Ueguchi, C., and T. Mizuno. 1993. The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. **12:**1039-1046.

Ussery, D.W., J.C.D. Hinton, B.J.A.M. Jordi, P.E. Granum, A. Seirafi, R.J. Stephen, A.E. Tupper, G. Berridge, J.M. Sidebotham, and C.F. Higgins. 1994. The chromatin-associated protein H-NS. Biochimie. **76:**968-980.

Valvano, M.A., and J.H. Crosa. 1984. Aerobactin iron transport genes commonly encoded by certain Col V plasmids occur in the chromosome of a human invasive strain of *Escherichia coli*. Infect. Immun. **46:**159-167.

Valvano, M.A., R.P. Silver, and J.H. Crosa. 1986. Occurrence of chromosome- or plasmid-mediated aerobactin iron transport systems and hemolysin production among clonal groups of human invasive strains of *Escherichia coli* K1. Infect. Immun. **52:**192-199.

Vicente, M., S.R. Kushner, T. Garrido, and M. Aldea. 1991. The role of the "gearbox" in the transcription of essential genes. Mol. Microbiol. **5:**2085-2091.

Vizán, J.L., C. Hernández-Chico, I. del Castillo, and F. Moreno. 1991. The peptide antibiotic microcin B17 induces double-stranded cleavage of DNA mediated by *E. coli* DNA gyrase. EMBO J. **10:**467-476.

Waalwijk, C., J.F. van den Bosch, D.M. MacLaren, and J. de Graaf. 1982. Plasmid content and virulence properties of urinary *Escherichia coli* strains. FEMS Microbiol. Lett. **14:**171-175.

Walker, J.E., M. Sarste, M.J. Runswick, and N.J. Gay. 1982. Distanly related sequences in the α- and β-subunits of ATP synthase, kinases and other ATP-binding enzymes and a common nucleotide binding fold. EMBO J. **1:**945-951.

Wallace, R.B., M.J. Johnson, S.V. Suggs, K. Miyoshi, R. Bhatt, and K. Itakura. 1981. A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector pBR322. Gene. **16:**21-26.

Wallis, R., A. Reilly, K. Barnes, C. Abell, D. Campbell, G.R. Moore, R. James, and C. Kleanthous. 1994. Tandem overproduction and characterization of the nuclease domain of colicin E9 and its cognate inhibitor protein Im9. Eur. J. Biochem. **220:**447-454.

Wandersman, C. 1992. Secretion across the bacterial outer membrane. TIG. **8:**317-322.

Wandersman, C., and P. Delepelaire. 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA. **87:**4776-4780.

Wandersman, C., and S. Létoffé. 1993. Involvement of lipopolysaccharide in the secretion *of Escherichia coli* α-hemolysin and *Erwinia chrysanthemi* proteins. Mol. Microbiol. **7:**141-150.

Wang, G., C.M. Berg, J.-M. Chen, A.C. Young, R.W. Blakesley, L.Y. Lee, and D.E. Berg. 1993a. Creating nested deletions for sequencing cosmid DNAs. FOCUS. **15:**47-49.

Wang, G., R.W. Blakesley, D.E. Berg, and C.M. Berg. 1993b. pDUAL: A transposon-based cosmid cloning vector for generating nested deletions and DNA sequencing templates in vivo. Proc. Natl. Acad. Sci. USA. **90:**7874-7878.

Warner, P.J., P.H. Williams, A. Bindereif, and J.B. Neilands. 1981. ColV plasmid-specific aerobactin synthesis by invasive strains of *Escherichia coli.* Infect. Immun. **33:**540-545.

Waters, V.L., and J.H. Crosa. 1991. Colicin V virulence plasmids. Microbiol. Rev. **55:**437-450.

Waters, V.L., and J.H. Crosa. 1986. DNA environment of the aerobactin iron uptake system genes in prototypic ColV plasmids. J. Bacteriol. **167:**647-654.

Webster, R.E. 1991. The *tol* gene products and the import of macromolecules into *Escherichia coli.* Mol. Microbiol. **5:**1005-1011.

Williams, P.H. 1979. Novel iron uptake system specified by ColV plasmids: an important component of the virulence of invasive strains of *Escherichia coli.* Infect. Immun. **26:**925-932.

Williams, P.H., and P.J. Warner. 1980. ColV plasmid-mediated colicin V-independent iron uptake system of invasive strains of *Escherichia coli.* Infect. Immun. **29:**411-416.

Wise, A., R. Brems, V. Ramakrishnan, and M. Villarejo. 1996. Sequences in the -35 region of *Escherichia coli rpoS*-dependent genes promote transcription by $E\sigma^S$. J. Bacteriol. **178:**2785-2793.

Wooley, R.E., L.K. Nolan, J. Brown, P.S. Gibbs, and D.I. Bounous. 1994. Phenotypic expression of recombinant plasmids pKT107 and pHK11 in an avirulent *Escherichia coli.* Avian Dis. **38:**127-134.

Yamada, H., T. Yoshida, K. Tanaka, C. Sasakawa, and T. Mizuno. 1991. Molecular analysis of the *Escherichia coli hns* gene encoding a DNA binding protein, which preferentially recognises curved DNA sequences. Mol. Gen. Genet. **230:**332-336.

Yamashino, T., C. Ueguchi, and T. Mizuno. 1995. Quantitative control of the stationary phase-specific sigma factor, σ^S, in *Escherichia coli*: Involvement of the nucleoid proteins H-NS. EMBO J. **14:**594-602.

Yang, C.C., and J. Konisky. 1984. Colicin V-treated *Escherichia coli* does not generate membrane potential. J. Bacteriol. **158:**757-759.

Yanisch-Perron, C., C. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of M13mp18 and pUC19 vectors. Gene. **33:**103-119.

Yorgey, P., J. Davagnino, and R. Kolter. 1993. The maturation pathway of microcin B17, a peptide inhibitor of DNA gyrase. Mol. Microbiol. **9:**897-905.

Yorgey, P., J. Lee, J. Kördel, E. Vivas, P. Warner, D. Jebaratnam, and R. Kolter. 1994. Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor. Proc. Natl. Acad. Sci. USA. **91:**4519-4523.

Zabarovsky, E.R., and G. Winberg. 1990. High efficiency electroporation of ligated DNA into bacteria. Nucleic Acids Res. **18:**5912.

Zhang, A., and M. Belfort. 1992. Nucleotide sequence of a newly-identified *Escherichia coli* gene *stpA*, encoding an H-NS-like protein. Nucleic Acids Res. **20:**6735.

Zhang, A., S. Rimsky, M.E. Reaban, H. Buc, and M. Belfort. 1996. *Escherichia coli* protein analogues StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. EMBO J. **15:**1340-1349.

Zhang, L.H., M.J. Fath, H.K. Mahanty, P.C. Tai, and R. Kolter. 1995. Genetic analysis of the colicin V secretion pathway. Genetics. **141:**25-32.

Zuber, F., D. Kotlarz, S. Rimsky, and H. Buc. 1994. Modulated expression of promoters containing upstream curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. Mol. Microbiol. **12:**231-240.

APPENDICES.

APPENDIX 1. BUFFERS AND SOLUTIONS.

0.5M EDTA.

18.612g	EDTA	0.5M	EDTA

Distilled water to 100ml pH 8.0.

5mM EDTA.

1ml	0.5M EDTA	5mM	EDTA

Distilled water to 100ml pH 8.0.

2M NaOH.

8g	NaOH	2M	NaOH

Distilled water to 100ml.

10% SDS.

50g	SDS	10%	SDS

Distilled water to 500ml.

50x TAE.

242g	Trizma Base	2M	Tris-acetate
100ml	0.5M EDTA pH 8.0	0.05M	EDTA
57.1ml	Glacial acetic acid		

Distilled water to 1 litre pH 8.0.

1x TAE.

40ml	50x TAE	1x	TAE

Distilled water to 2 litres.

$T_{10}E_1$ (TE).

10mM	Tris-HCl pH 8.0
1mM	EDTA pH 8.0

10x TBE.

121.1g	Trizma base	1M	Tris
51.35g	Boric acid	0.83M	Borate
3.72g	EDTA $Na_2 2H_2 0$	10mM	EDTA

Distilled water to 1 litre pH 8.0.

SSC SOLUTIONS.

20x SSC.

350.6g	NaCl	3M	NaCl
176.4g	Na_3Citrate	0.3M	Na_3Citrate

Distilled water to 2 litres, pH 7.0. Adjust pH with HCl.

2x SSC.

100ml	20x SSC	2x	SSC

distilled water to 1 litre.

0.1x SSC.

5ml	2x SSC	0.1x	SSC

distilled water to 100ml.

LOADING BUFFER FOR AGAROSE GELS.

30%	Glycerol
0.25%	Bromophenol blue
0.25%	Xylene cyanol

RNaseA to a final concentration of 10ug/ml was added when required.

5x LIGATION BUFFER (STICKY-END, BRL).

250mM	Tris-HCl pH 7.6
50mM	$MgCl_2$
5mM	ATP
5mM	DTT
25 %(w/v)	PEG-8000

ALKALINE EXTRACTION OF DNA.

Solution I.

50mM	Glucose
10mM	EDTA
25mM	Tris HCl pH 8.0
2mg/ml	Lysozyme

Solution II.

1%	SDS
0.2M	NaOH

Solution III.

3M	NaAcetate pH 4.8

LARGE SCALE PLASMID PREPARATION.

Solution I.

1%	Glucose
25mM	Tris-HCl pH 8.0
10mM	EDTA

Solution II.

0.2%	NaOH
1%	SDS

Solution III.

5M	KAcetate

5M LiCl.

21.2g	LiCl	5M	LiCl

Distilled water to 100ml.

2.5M NaCl/20%PEG.

14.61g	NaCl	2.5M	NaCl
20ml	Polyethylene glycol	20%	PEG

Distilled water to 100ml.

DEPHOSPHORYLATION USING CIP.

0.12g	Tris-HCl	10mM	Tris-HCl

Distilled water to 100ml. Adjust to pH 8.3 with HCl.

ELECTROPORATION.

10ml	100% Glycerol	10%	Glycerol

Distilled water to 100ml.

ELECTROPORATION OF LIGATED DNA.

9.814g	KAcetate	1M	KAacetate

Distilled water to 100ml. Adjust to pH 8.0 with

PREPARATION OF COMPETENT CELLS.

Solution I.

10mM	NaCl

Solution II.

100mM	$CaCl_2$

MEASURING B-GALACTOSIDASE ACTIVITY.

1x A medium.

10.5g	K_2HPO_4
4.5g	KH_4PO_4
1.0g	$(NH_4)_2SO_4$
0.5g	$NaCitrate.2H_2O$

Distilled water to 1l. Autoclave.

Z Buffer.

0.06M	$Na_2HPO_4.7H_2O$
0.04M	$NaH_2PO_4.H_2O$
0.01M	KCl
0.001M	$MgSO_4.7H_2O$
0.05M	β-mercaptoethanol

pH = 7. Do not autoclave.

1M Na_2CO_3.

105.99g	Na_2CO_3	1M Na_2CO_3

Distilled water to 1litre. Do not autoclave.

SDS-PAGE PROTEIN GEL STOCK SOLUTIONS.

Monomer Solution (30%T 2.7%CBis).

58.4g	Acrylimide	30%	Acrilimide
1.6g	Bis	7%	Bis

Distilled Water to 200ml.

Resolving Gel Buffer.

36.3g	Tris-HCl	1.5M	Tris-HCl

Distilled water to 200ml.

Adjust to pH 8.8 with HCl.

Stacking Gel Buffer.

3.0g	Tris-HCl	0.5M	Tris-HCl

Distilled water to 50ml.

Adjust to pH 6.8 with HCl.

Initiator.

0.5g	Ammonium persulphate (APS)	10%	Ammonium persulphate

Distilled water to 5ml.

Resolving Gel Overlay.

25ml	1.5M TrisHCl	0.375M	Tris-HCl
1.0ml	10% SDS	0.1%	SDS

Distilled water to 100ml pH 8.8.

2x Treatment Buffer.

2.5ml	0.5M Tris-HCl	0.125M	Tris-HCl
4.0ml	10% SDS	4%	SDS
2.0ml	Glycerol	20%	Glycerol
1.0ml	2-mercaptoethanol	10%	2-mercaptoethaol

Distilled water to 10ml pH 6.8.

Tank Buffer.

12g	Tris-HCl	0.025M	Tris-HCl
57.6g	Glycine	0.192M	Glycine
40ml	10% SDS	0.1%	SDS

Distilled water to 4 litres pH 8.3.

Stain Stock.

2.0g	Coomassie Blue R-250	1%	Coomassie brilliant blue R-250

Distilled water upto 200ml. Stir and filter.

Stain.

62.5ml	Stain stock	0.125%	Coomassie brilliant blue R-250
250ml	100% Methanol	50%	Methanol
50ml	100% Acetic acid	10%	Acetic acid

Distilled water to 500ml.

Destaining Solution 1.

500ml	100% Methanol	50%	Methanol
100ml	100% Acetic acid	10%	Acetic acid

Distilled water to 1 litre.

Destaining Solution 2.

500ml	100% Methanol	5%	Methanol
700ml	100% Acetic acid	7%	Acetic acid

Distilled water to 10 litres.

SDS-PAGE Protein Gel Recipes - 1.5mm thick slab gel.

	Separating Gel			Stacking Gel
	Uniform 15%	*Gradient* 10%	10%-20% 20%	4%T 2.7%C
30%T 2.7%C	15.00ml	5.00ml	10.00ml	1.33ml
Separating Gel Buffer	7.50ml	3.75ml	3.75ml	
Stacking Gel Buffer				2.50ml
10% SDS	0.30ml	0.150ml	0.150ml	1.00ml
Distilled water	7.00ml	6.00ml	1.00ml	6.10ml
10% APS	0.10ml	0.05ml	0.05ml	0.05ml
TEMED	0.025ml	0.0125ml	0.0125ml	0.025ml
Total volume	30.00ml	15.00ml	15.00ml	10.00ml

Degas first four components prior to adding 10% APS and TEMED.

Pour a layer of water-saturated butanolover the separating gel to aid polymerisation. Remove and rinse before stacking gel is poured.

SILVER STAINING.

Wash 1.

500ml	100% Methanol Stock	50%	Methanol
100ml	100% Acetic acid Stock	10%	Acetic acid

Distilled water to 1 litre.

Wash 2.

80ml	25% Glutaraldehyde Stock	10%	Glutaraldehyde

Distilled water to 200ml.

NaOH Stock.

9ml	1M NaOH	0.36%	NaOH

Distilled water to 100ml.

Aqueous Silver Nitrate Stock.

0.2g Silver Nitrate crystals per ml Distilled water.

Developer Solution.

10ml	100% Ethanol
2.5ml	1% Citric acid
0.25ml	38% Formaldehyde Stock Solution

Distilled water to 1 litre.

Fixer Solution.

50ml	100% Acetic acid	5%	Acetic acid

Distilled water to 1 litre.

Staining Solution.

Add in order with stirring.

15.3ml	0.36% NaOH
1.03ml	Concentrated NH_4OH. Added dropwise.
3.0ml	Silver Nitrate Stock. Added dropwise.
55.0ml	100% Ethanol. Added slowly with a Pasteur pipette.

If at anytime the solution went brown while adding Silver Nitrate or Ethanol,

the solution was carefully back titrated with concentrated NH_4OH until clear.

SDS-PAGE STOCK SOLUTIONS FOR RESOLUTION OF LOW MOLECULAR WEIGHT PROTEINS (Thomas and Kornberg, 1978).

Separating Gel Buffer.

72.7g	Tris-HCl	3M	Tris-HCl

Distilled water to 200ml

Adjust to pH 8.8 with HCl.

Separating Gel Acrylamide/Bis Solution.

75ml	40% Acrylamide stock	30%	Acrylamide
7.5ml	2% Bisacrylamide stock	0.15%	Bisacrylamide

Distilled water to 100ml.

Stacking Gel Buffer.

6g	Tris-HCl	0.5M	Tris-HCl

Distilled water to 100ml

Adjust to pH 6.8 with HCl.

Stacking Gel Acrylamide/Bis Solution.

2.5ml	40% Acrylamide solution	10%	Acrylamide
2.5ml	2% Bisacrylamide	0.5%	Bisacrylamide

Distilled water to 10ml.

5x Stock Tank Buffer.

30.2g	Tris-HCl	50mM	Tris-HCl
144g	Glycine	0.38M	Glycine
50ml	10% SDS	0.1%	SDS

Distilled water to 1 litre.

Predying Solution.

400ml	100% Methanol	40%	Methanol
100ml	100% Acetic Acid	10%	Acetic acid
30ml	100% Glycerol	3%	Glycerol

Distilled water to 1 litre.

SDS-PAGE Protein Gel Recipes.

Solution	Separating gel	Stacking gel
Acrylamide:Bis (30%:0.15)	30ml	
Acrylamide:Bis (10%:0.5%)		6ml
Tris-HCl 3M pH8.8	12.5ml	
Tris-HCl 0.5M pH 6.8		4.8ml
Distilled Water	6.25ml	8.8ml
TEMED	0.01ml	0.01ml
10% SDS	0.5ml	0.2ml
10% Ammonium persulphate	0.5ml	0.2ml

Degas mixture of first four components prior to adding add 10% SDS and 10% APS prior to pouring gel.

PREPARATION AND LABELLING OF MINI-CELLS.

20% Sucrose/M63.

20g Sucrose 20% Sucrose

1x M63 salts to 100ml.

BSG Buffer.

8.5g	NaCl	8.5g/l	NaCl
0.3g	KH_2PO_4	0.3g/l	KH_2PO_4
0.6g	Na_2HPO_4	0.6g/l	Na_2HPO_4
0.1g	Gelatin	0.1g/l	Gelatin

Distilled water to 1 litre pH 7.4. Adjust pH with KOH.

Storage Buffer.

0.7g	Na_2HPO_4	7g/l	Na_2HPO_4
0.3g	KH_2PO_4	0.3g/l	KH_2PO_4
0.4g	NaCl	4g/l	NaCl
0.01g	$MgSO_4$	0.1g/l	$MgSO_4$

Distilled water to 100ml.

MAXICELLS.

0.4g	Glucose	0.4%	Glucose

1x M63 salts to 100ml.

AMMONIUM SULPHATE PRECIPITATION.

6.8g	KH_2PO_4	50mM	KH_2PO_4

Distilled water to 1 litre.

BULK PREPARATION OF MICROCIN 24 BY NITROCELLOLOSE EXTRACTION.

Stock Solutions.

1.14g	Na_2HPO_4	0.1M	Na_2HPO_4

Distilled water to 100ml.

1.56g	NaH_2PO_4	0.1M	NaH_2PO_4

Distilled water to 100ml.

Phosphate Buffer (Wash Buffer).

77.4ml	0.1M Na_2HPO_4	0.1M	Phosphate
22.6ml	0.1M NaH_2PO_4	0.1M	Phosphate

pH 7.4.

Elution Buffer.

1ml	Triton X-100	1%	Triton
1ml	0.5M EDTA	5mM	EDTA

Wash buffer to 100ml.

GEL FILTRATION.

Buffer.

6.8g	KH_2PO_4	50mM	KH_2PO_4

Distilled water to 1 litre, pH 6.5. Adjust pH with KOH.

Column.

15g	Sephadex G-100	12-15x	Volume

Soaked in excess buffer for 24 hours.

Column Wash.

8g	NaOH	0.2M	NaOH

Distilled water to 1 litre.

Column Storage.

0.02g	Sodium Azide	0.02%	Sodium Azide

Buffer to 100ml.

ION EXCHANGE CHROMATOGRAPHY.

Start Buffers.

11.92g	HEPES	50mM	HEPES

Distilled water to 1 litre. Adjust to pH 7.6 with NaOH. Use with SP column.

2.42g	Tris-HCl	20mM	Tris-HCl

Distilled water to 1 litre. Adjust to pH 8.2 with HCl. Use with Q column.

Elution Buffer.

5.84g	NaCl	1M	NaCl

Start buffer to 100ml.

Storage Buffers.

20ml	100% Ethanol	20%	Ethanol
6.67ml	3M NaAcetate pH 4.3	0.2M	NaAcetate

Distilled water to 100ml. Use with SP column.

20ml	100% Ethanol	20% Ethanol

Distilled water to 100ml. Use with Q column.

NUCLEASE ACTIVITY ASSAY.

Stock solutions.

121.1g	Tris-HCl	1M	Tris-HCl

Distilled water to 1 litre. Adjust to pH 8.0 with HCl.

29.22g	NaCl	5M	NaCl

Distilled water to 100ml.

24.65g	$MgSO_4$	1M	$MgSO_4$

Distilled water to 100ml.

Reaction Buffer.

2ml	1M Tris-HCl pH 8.0	20mM	Tris-HCl
1.6ml	5M NaCl	80mM	NaCl
1.0ml	1M $MgSO_4$	20mM	$MgSO_4$

Distilled water to 10ml.

Dilution Buffer.

1.25ml	Glycerol	12.5%	Glycerol
100µl	0.5M EDTA	5mM	EDTA
100µl	20% SDS	0.2%	SDS
0.001g	Bromophenol Blue	0.01%	Bromophenol Blue

Distilled water to 10ml.

APPENDIX 2. MEDIA.

H-TOP AGAR.

10g	Bacto-tryptone	1%	Tryptone
8g	NaCl	0.8%	NaCl
8g	Agar	0.8%	Agar

Distilled water to 1 litre.

LURIA-BERTANI MEDIA (LB).

10g	Bacto-tryptone	1%	Tryptone
5g	Yeast extract	0.5%	Yeast extract
5g	NaCl	0.5%	NaCl

Distilled water to 1 litre pH 7.0 - 7.4.

LB AGAR.

As above plus

15g	Agar	1.5%	Agar
20g	Agar	2.0%	Agar

2xYT MEDIA.

10g	Bacto-tryptone	1%	Tryptone
10g	Yeast extract	1%	Yeast extract
5g	NaCl	0.5%	NaCl

Distilled water to 1 litre.

SOC (Electroporation media).

2g	Bact-tryptone	2%	Typtone
0.5g	Yeast extract	0.5%	Yeast Extract
200ml	5M NaCl	10mM	NaCl
250ml	1M KCl	2.5mM	KCl
1ml	1M $MgCl_2$	10mM	$MgCl_2$
10ml	0.1 M $MgSO_4$	10mM	$MgSO_4$
2ml	1M Glucose	20mM	Glucose

Distilled water to 100 ml.

SUPER BROTH.

16g	Bact-Tryptone	1.6%	Tryptone
10g	Yeast Extract	1.0%	Yeast Extract
2.5g	NaCl	0.25%	NaCl

Distilled water to 1 litre.

TBMM.

10g	Bacto-tryptone	1%	Tryptone
5g	NaCl	0.5%	NaCl

Distilled water to 980ml.

Autoclave.

Add from sterile stock:

10ml	20% Maltose	0.2%	Maltose
12.5ml	20% $MgSO_4$	10mM	$MgSO_4$
100Ml	1% Thiamine	1Mg/ml	Thiamine

TRYPTONE AGAR.

10g	Bacto-tryptone	1%	Tryptone
8g	NaCl	0.8%	NaCl
20g	Agar	2%	Agar

Distilled water to 1 litre.

M63 MEDIA.

1x M63 SALTS

13.6g	KH_2PO_4	0.1M	KH_2PO_4
2g	$(NH_4)_2SO_4$	0.015M	$(NH_4)_2SO_4$
0.5mg	$FeSO_4.7H_2O$	$1.8x10^{-6}$M	$FeSO_4.7H_2O$

Distilled water to 1 litre and autoclave.

Add/ litre:

1ml	20% $MgSO_4$	0.02%	$MgSO_4$
0.5ml	1% Thiamine-HCl	0.0005%	Thiamine-HCl
10ml	20% Glucose	0.2%	Glucose

M63 / MINIMAL AGAR (MA).

40g	Agar	2%	Agar

Distilled water to 1 litre and autoclave.

Add to 1 litre 1x M63 salts. When cooled sufficiently add $MgSO_4$, Thiamine and Glucose

AMINO ACID SUPPLEMENTS FOR MINIMAL MEDIA (Rodriguez and Tait, 1983).

Amino acid	100x stock solution (mg/ml)
L-alanine	2
L-arginine	2
L-asparagine	2 *
L-aspartic acid	10 *
L-cysteine	2 *
glycine	2
L-glutamine	2
L-glutamic acid	10
L-histidine (free base)	2
L-isoleucine	3
L-leucine	3
L-lysine	3
l-methionine	2
L-phenylalanine	5 in 0.01N HCl
L-proline	2
L-serine	37.5
L-threonine	20
L-tryptophane	2 *
L-tyrosine	3 * in 10mM NaOH
L-valine	10

*. Filter sterilise. All others may be autoclaved. Amino acids dissolved in double distilled water unless stated.

APPENDIX 3. PRIMERS USED FOR DIDEOXYNUCLEOTIDE SEQUENCING.

Primer	Sequence (5'-3')[a]	Reference
T7	-TAATACGACTCACTATAGGG-	Chen and Seeburg, 1985
T3	-ATTAACCCTCACTAAAGGGA-	Chen and Seeburg, 1985
Sp6	-GATTTAGGTGACACTATAG-	Chen and Seeburg, 1985
*Bam*HIcw	-CACTATCGACTACGCGATCA-	Wallace, 1981
*Bam*HIccw	-ATGCGTCCGGCGTAGA-	Wallace, 1981
Mini-Tn*10lacZ'*	-ACGACGTTGTAAAACGACGG-	This study
Mini-Tn*10tetA*	-AAGGCACCTTTGGTCACCAA-	This study
MKBGAL1	-AAGGGCGATCGGTGCGGGCCTCTTC-	M. Kennedy
813 T32	186-TTTTGAGCAGTTGCTCGAAA-205	This study
Primer R5	838-TAATAAGTTAACAAGAATGA-857	This study
813 K70[b]	1120-TCTCTCATATACATCTCCTG-1139	This study
851 K5[b]	2879-TCCGGGATAGGTCTGCATTT-2898	This study
851 K5a[b]	2700-TTATTTCTTCTGGAATAATT-2719	This study
851 T22	2241-AACTACACTACAGACACTGG-2260	This study
851 T22a	2388-CATAAACAAAGACCAGTTAA-2407	This study

a. Nucleotide numbers relate to coding strand.

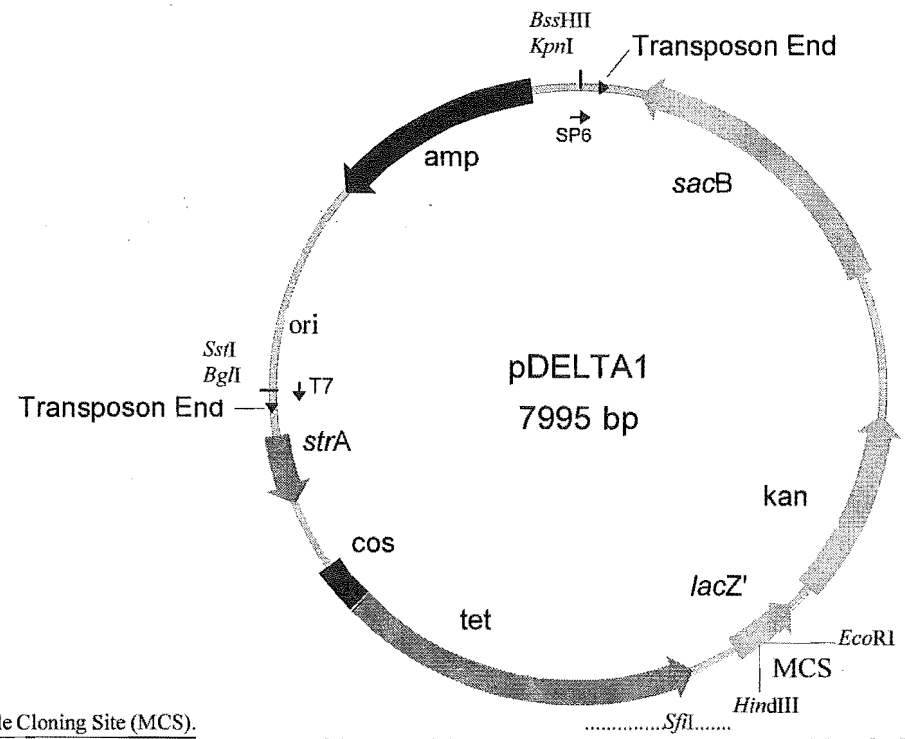
b. Complementary strand primers.

APPENDIX 4. PLASMIDS CREATED FOR SEQUENCING.

The pUC18 fragment in pGΔ185 and pGΔ851 corresponds to bases 5'-3690-3740-3' of pBR322.

Restriction Enzymes: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; R, *Eco*RV; S, *Sal*I; Sa, *Sau*3AI;

A. pDELTA1 (7.995 kb, Deletion Factory™ system, BRL).



Multiple Cloning Site (MCS).

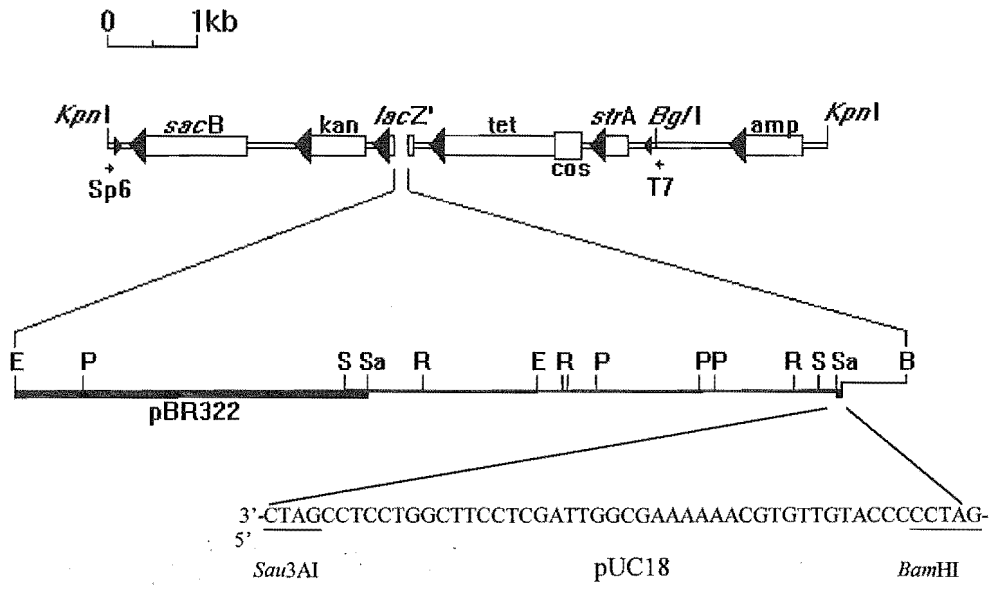
5'-TTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCCCGGGCCATGGAGGCCACGCGTGCGGCCGC

*Eco*RI *Sma*I *Nco*I *Mlu*I *Not*I

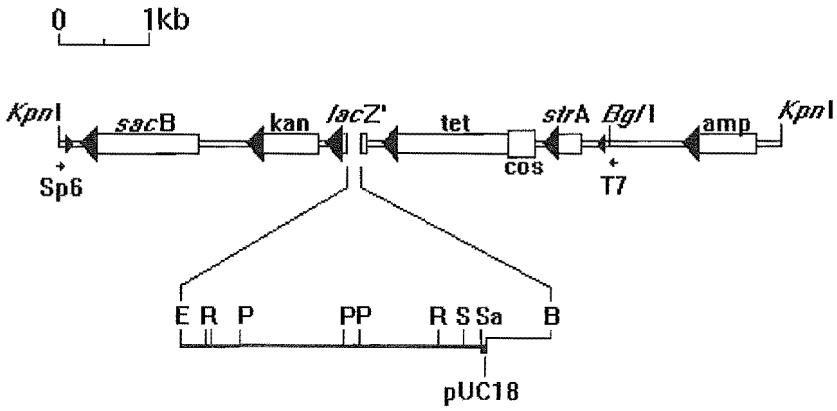
GGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT-3'

*Bam*HI *Xba*I *Sal*I *Pst*I *Sph*I *Hin*dIII *Sfi*I

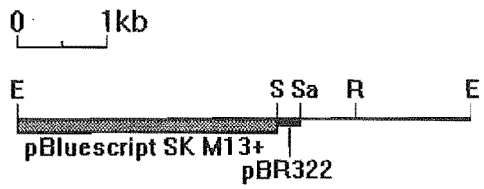
B. pGΔ185 (17.258 kb)



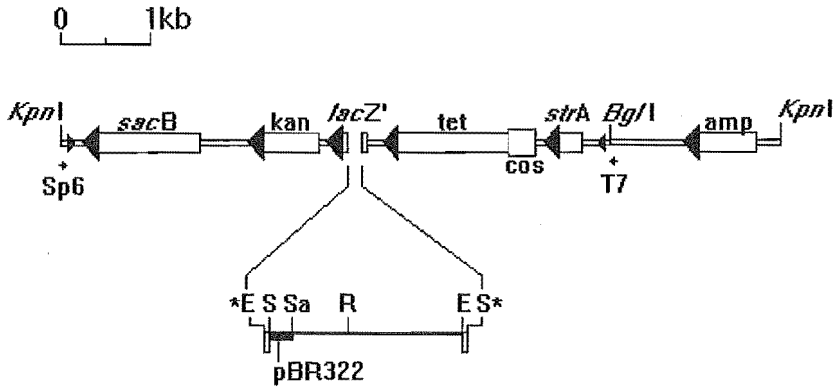
C. pGΔ851 (11.361 kb)



D. pGSK813 (5.113 kb).



E. pGΔ813 (10.24 kb)



5'-GAATTCCCGGGCCATGGAGGCCACGCGTGCGGCCGCGGATCCTCTAGAGTCGAC-3'
EcoRI BamHI SalI

Duplication of pDELTA1 MCS.

APPENDIX 5. RESTRICTION ENZYME SITES IN pGOB18 SEQUENCE.

ENZYME NAME	SEQUENCE	CUTTING POSITION	TOTAL
*Alu*I	AG!CT	217 636 642 1328 1535 1687 2288 2584 3678 4609 4929 4933	12
*Apa*I	GGGCC!C	1244	1
*Ava*I	C!YCGRG	3355 5038	2
*Bgl*I	GCCNNNN!NGGC	3247	1
*Bst*BI	TT!CGAA	3602	1
*Bst*EII	G!GTNACC	2488	1
*Eco*RI	G!AATTC	1908	1
*Eco*RV	GAT!ATC	617 2182 2212 4784	4
*Hae*II	RGCGC!Y	5046 5217	2
*Hae*III	GG!CC	750 1242 2026 2804 2826 2867 3100 3219 3241 3554 3748 4560 4695 5082	14
*Hpa*II	C!CGG	54 136 318 335 1337 1727 1904 2663 2864 2895 2954 3180 3480 3532 3556 3590 3632 3878 4021 4544 4581 4668 4690 4756 5058	25
*Kpn*I	GGTAC!C	2747 4780	2
*Msp*I	C!CGG	54 136 318 335 1337 1727 1904 2663 2864 2895 2954 3180 3480 3532 3556 3590 3632 3878 4021 4544 4581 4668 4690 4756 5058	25
*Nde*I	CA!TATG	1332	1
*Nhe*I	G!CTAGC	4929	1
*Pst*I	CTGCA!G	2562 3737 3892	3
*Rsa*I	GT!AC	68 560 697 1522 1981 2055 2143 2279 2745 3022 3269 3638 3968 4047 4194 4729 4747 4778	18
*Sal*I	G!TCGAC	5053	1
*Sau*3AI	!GATC	1 716 1180 1192 1611 3260 3333 3371 3476 3719 3740 4788 5264	13
*Taq*I	T!CGA	201 518 1021 2184 2300 3228 3602 3743 4860 5039 5054	11
*Xho*I	C!TCGAG	5038	1

! Cutting site

APPENDIX 6: BLAST SERVER SEQUENCE SIMILARITY COMPARISONS.

1. MdbA.

Sequences producing High-scoring Segment Pairs: Reading High Probability

Frame	Score	P(N)	N					
gi|1197456			(U47048) microcin DNA binding pro...	+1	855	2.1e-113	1
sp|P30017|STPA_ECOLI			DNA-BINDING PROTEIN STPA. >pir||J...	+1	169	9.5e-36	2
sp|P08936|HNS_ECOLI			DNA-BINDING PROTEIN H-NS (HISTONE...	+1	155	1.7e-31	3
pir||S23868			gene h-ns protein - Escherichia c...	+1	155	3.3e-31	3
pir||S03772			DNA-binding protein H-NS - Escher...	+1	149	1.1e-30	3
sp|P18955|HNS_SERMA			DNA-BINDING PROTEIN H-NS (HISTONE...	+1	151	1.4e-29	3
sp|P18818|HNS_PROVU			DNA-BINDING PROTEIN H-NS (HISTONE...	+1	157	7.2e-28	3
gi|154220			(M37891) histone H1-like protein ...	+1	157	1.5e-24	2
sp|P17428|HNS_SALTY			DNA-BINDING PROTEIN H-NS (HISTONE...	+1	157	1.5e-24	2
sp|P43841|HNS_HAEIN			DNA-BINDING PROTEIN H-NS HOMOLOG....	+1	114	1.2e-21	2
gi|47036			(X13131) kcpA gene product (AA 1 ...	+1	110	1.9e-21	3
pdb|1HNR|			H-Ns (Dna-Binding Domain) (Nmr, M...	+3	76	3.0e-06	2
gi|212879			(K02113) vitellogenin [Gallus gal...	-3	88	0.0043	1
sp|P02845|VIT2_CHICK			VITELLOGENIN II PRECURSOR (CONTAI...	-3	88	0.0057	1
gi|212881			(M18060) vitellogenin [Gallus gal...	-3	88	0.0057	1
sp|P40552|YIB1_YEAST			HYPOTHETICAL 26.3 KD PROTEIN IN B...	-3	81	0.044	1
gi|1197667			(U46857) vitellogenin [Anolis pul...	-3	79	0.071	1
sp|P42505|HVRA_RHOCA			TRANS-ACTING REGULATORY PROTEIN H...	+3	77	0.082	1
gi|1197665			(U46856) vitellogenin [Anolis pul...	-3	77	0.14	1
sp|P32323|AGA1_YEAST			A-AGGLUTININ ATTACHMENT SUBUNIT P...	-3	77	0.18	1
pir||PQ0759			polyprotein - turnip mosaic virus...	+1	76	0.23	1
gi|295671			(L11275) selected as a weak suppr...	-3	75	0.30	1
sp|P32583|SR40_YEAST			SUPRESSOR PROTEIN SRP40. >pir||S3...	-3	75	0.30	1
sp|P28738|KINH_MOUSE			KINESIN HEAVY CHAIN. >pir||S37711...	+1	75	0.33	1
gi|516516			(U06698) neuronal kinesin heavy c...	+1	75	0.33	1
prf||2113194A			H ATPase inhibitor [Solanum tuber...	+1	66	0.33	1
gi|459202			(U07055) vitellogenin 1 [Fundulus...	-3	75	0.33	1
pir||S34257			hypothetical protein 4 - Escheric...	+3	64	0.35	2
pir||A48347			coat protein - turnip mosaic viru...	+1	74	0.38	1
gi|940016			(U25663) coat protein [Turnip mos...	+1	74	0.39	1
pir||S11689			coat protein - turnip mosaic viru...	+1	74	0.39	1
gi|62047			(X52804) coat protein [Turnip yel...	+1	74	0.39	1
sp|P40442|YIQ9_YEAST			HYPOTHETICAL 99.7 KD PROTEIN IN S...	-3	74	0.42	1
pir||C61615			sericin MG-2 - greater wax moth (...	-3	72	0.59	1
pir||S21499			coat protein - turnip mosaic virus	+1	72	0.59	1
gi|535078			(X65978) coat protein [Turnip mos...	+1	72	0.59	1
pir||S48795			troponin T - human >gi|587434 (X7...	+1	71	0.65	1
gi|1200154			(X95759) glycogen (starch) syntha...	+1	72	0.66	1
sp|P02567|MYSD_CAEEL			MYOSIN HEAVY CHAIN D (MHC D). >pi...	+1	72	0.66	1
pir||PQ0757			polyprotein - turnip mosaic virus...	+1	71	0.73	1
sp|P14328|SP96_DICDI			SPORE COAT PROTEIN SP96. >pir||S0...	-3	71	0.75	1
pir||S47179			hypothetical protein - common tob...	+1	54	0.78	3
pir||A33513			hypothetical protein B - human T-...	+1	61	0.79	2
gi|746442			(U23455) No definition line found...	+1	59	0.81	1
pir||S51329			coat protein - turnip mosaic viru...	+1	70	0.82	1
gi|669069			(Z48241) C32A3.2 [Caenorhabditis ...	+1	70	0.83	1
gi|1072358			(Z68108) T05A10.3 [Caenorhabditis...	+1	70	0.84	1
sp|P42568|AF9_HUMAN			AF-9 PROTEIN. >gi|306449 (L13744)...	-3	70	0.85	1
sp|Q02224|CENE_HUMAN			CENTROMERIC PROTEIN E (CENP-E PRO...	+1	70	0.88	1
sp|P26686|SR55_DROME			SERINE-ARGININE PROTEIN 55 (SRP55...	-3	62	0.91	2
gi|1072217			(U40946) coded for by C. elegans ...	+1	69	0.92	1
sp|P11837|NIMA_EMENI			G2-SPECIFIC PROTEIN KINASE. >pir|...	+1	49	0.94	3
sp|P40480|YIL2_YEAST			HYPOTHETICAL 123.6 KD PROTEIN IN ...	+1	69	0.94	1
gi|171959			(L01992) myosin-like protein [Sac...	+1	69	0.94	1
sp|Q02455|MLP1_YEAST			MYOSIN-LIKE PROTEIN MLP1. >pir||S...	+1	69	0.94	1
gi|144666			(M37402) [Plasmid ColA, complete ...	+1	67	0.97	1
sp|P01154|POPI_BOVIN			POSTERIOR PITUITARY PEPTIDE. >pir...	+3	38	0.97	2
gi|1103869			(U39815) surface coat glycoprotei...	-3	67	0.98	1
pir||S28974			vitellogenin precursor - silver l...	-3	68	0.98	1
pir||JQ1168			polyprotein - turnip mosaic virus...	+1	68	0.98	1
sp|P19649|TMPB_TREPA			TREPONEMAL MEMBRANE PROTEIN B PRE...	+1	64	0.98	2
pir||C43592			outer membrane protein TmpB - Tre...	+1	64	0.98	2
sp|Q02597|POLG_TUMVQ			GENOME POLYPROTEIN (CONTAINS: N-T...	+1	68	0.98	1
sp|P30427|PLEC_RAT			PLECTIN. >pir||S21876 plectin - r...	+1	71	0.998	2
gi|452945			(S67069) autoantigen {clone R1} [...	+1	66	0.999	1
gi|437639			(L04159) [Plasmodium falciparum 3...	+1	66	0.9991	1
sp|Q01565|GSQD_ERWCH			GENERAL SECRETION PATHWAY PROTEIN...	-3	66	0.9993	1
sp|P13816|GARP_PLAFF			GLUTAMIC ACID-RICH PROTEIN. >pir|...	+1	66	0.9993	1
gi|544939			(S68736) myosin heavy chain, MHC ...	+1	66	0.9994	1
sp|P45387|HAP_HAEIN			ADHESION AND PENETRATION PROTEIN ...	+1	66	0.9995	1
gi|940233			(U32574) myosin heavy chain [Oryc...	+1	66	0.9995	1

```
prf||1819485A          CENP-E protein [Homo sapiens]          +1    66  0.9996    1
sp|Q06666|T2_MOUSE     OCTAPEPTIDE-REPEAT PROTEIN T2. >g...  +1    60  0.9996    2
pir||A35419            neutrophil protein - pig (fragmen...  -3    65  0.9999    1
gi|155172              (L08499) nitrogen regulator 1 [Vi...  +1    65  0.99991   1
sp|P19906|NTRB_VIBAL NITROGEN REGULATION PROTEIN NTRB.       +1    65  0.99991   1
pir||JL0114            ntrB protein - Vibrio alginolyticus   +1    65  0.99991   1
pir||S33068            myosin II heavy chain - fluke (Sc...  +1    65  0.99995   1
gi|11067               (X65591) myosin II heavy chain [S...  +1    65  0.99995   1


gi|1197456 (U47048) microcin DNA binding protein [Escherichia coli] Length = 171
Plus Strand HSPs: Score = 855 (393.3 bits), Expect = 2.1e-113, P = 2.1e-113
                  Identities = 171/171 (100%), Positives = 171/171 (100%), Frame = +1

   Query:     1 MSELTKEDEYGIISRTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAER 180
                MSELTKEDEYGIISRTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAER
   Sbjct:     1 MSELTKEDEYGIISRTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAER 60

   Query:   181 AERERKRQELLQLIAGEGFSPEELLGLSEEAPKSRKKTLPKPRLNISLKKMVKRNTGLVV 360
                AERERKRQELLQLIAGEGFSPEELLGLSEEAPKSRKKTLPKPRLNISLKKMVKRNTGLVV
   Sbjct:    61 AERERKRQELLQLIAGEGFSPEELLGLSEEAPKSRKKTLPKPRLNISLKKMVKRNTGLVV 120

   Query:   361 DVRQNQLLKHWQVVVLLMSFSSRNDSTALLFSGGLYPLKHTCQPILYKCYV 513
                DVRQNQLLKHWQVVVLLMSFSSRNDSTALLFSGGLYPLKHTCQPILYKCYV
   Sbjct:   121 DVRQNQLLKHWQVVVLLMSFSSRNDSTALLFSGGLYPLKHTCQPILYKCYV 171

>sp|P30017|STPA_ECOLI DNA-BINDING PROTEIN STPA. >pir||JH0774 stpA protein -
Escherichia coli >gi|43008 (X69210) H-NS-like protein [Escherichia
coli] >gi|469172 (U07823) H-NSB [Escherichia coli]. Length = 134
Plus Strand HSPs: Score = 169 (77.7 bits), Expect = 9.5e-36, Sum P(2) = 9.5e-36
                  Identities = 36/80 (45%), Positives = 52/80 (65%), Frame = +1

   Query:    34 IISRTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELL 213
                ++ +++ NIR+LR  ARE   +  L EM EK  VV +ERRE+ E++  E AER+ K    L
   Sbjct:     3 VMLQSLNNIRTLRAMAREFSIDVLEEMLEKFRVVTKERREEEEQQQRELAERQEKISTWL 62

   Query:   214 QLIAGEGFSPEELLGLSEEA 273
                +L+  +G +PEELLG S  A
   Sbjct:    63 ELMKADGINPEELLGNSSAA 82

Score = 42 (19.3 bits), Expect = 4.8e-13, Sum P(2) = 4.8e-13
Identities = 16/59 (27%), Positives = 26/59 (44%), Frame = +1

   Query:   130 VVIEERREDAEREAAERAERERKRQELLQLIAGEGFSPEELLGLSEEAPKSRKKTLPKP 306
                V  E R E+ +++       +E+    L  + A       E L   S  AP++ KK  P+P
   Sbjct:    36 VTKERREEEEQQQRELAERQEKISTWLELMKADGINPEELLGNSSAAAPRAGKKRQPRP 94

>sp|P08936|HNS_ECOLI DNA-BINDING PROTEIN H-NS (HISTONE-LIKE PROTEIN HLP-II)
(PROTEIN H1) (PROTEIN B1). >sp|P09120|HNS_SHIFL DNA-BINDING PROTEIN
 H-NS (PATHOGENESIS PROTEIN KCPA). >pir||S09325 DNA-binding protein
 drdx - Escherichia coli >pir||S00903 DNA-binding protein H-NS -
 Escherichia coli >pir||S23789 histone-like protein H-NS -
 Escherichia coli >pir||S24755 DNA-binding protein H-NS - Shigella
 flexneri >pir||S28633 DNA-binding protein H-NS - Escherichia coli
>gi|41736 (X07688) hns gene (AA 1-137) [Escherichia coli] >gi|42027
 (X59940) histone-like protein H-NS [Escherichia coli] >gi|42183
 (X57231) DNA-binding protein OsmZ [H-NS(H1a)] [Escherichia coli]
>gi|47070 (X66848) pid:g47070 [Shigella flexneri] >prf||1607341A
 drdX gene [Escherichia coli] Length = 137
Plus Strand HSPs: Score = 155 (71.3 bits), Expect = 1.7e-31, Sum P(3) = 1.7e-31
                  Identities = 34/71 (47%), Positives = 44/71 (61%), Frame = +1

   Query:    43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                + + NIR+LR  ARE   E L EM EKL VV+ ERRE+    AAE  ER RK Q+  +++
   Sbjct:     6 KILNNIRTLRAQARECTLETLEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYREML 65

   Query:   223 AGEGFSPEELL 255
                 +G  P ELL
   Sbjct:    66 IADGIDPNELL 76
```

```
>pir||S23868 gene h-ns protein - Escherichia coli >gi|43078 (X67326) H-ns[Escherichia
coli]
Length = 135
Plus Strand HSPs: Score = 155 (71.3 bits), Expect = 3.3e-31, Sum P(3) = 3.3e-31
                  Identities = 34/71 (47%), Positives = 44/71 (61%), Frame = +1

   Query:     43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                 + + NIR+LR  ARE   E L EM EKL VV+ ERRE+    AAE  ER RK Q+  +++
   Sbjct:      6 KILNNIRTLRAQARECTLETLEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYREML 65

   Query:    223 AGEGFSPEELL 255
                 +G  P ELL
   Sbjct:     66 IADGIDPNELL 76

>pir||S03772 DNA-binding protein H-NS - Escherichia coli Length = 136
Plus Strand HSPs: Score = 149 (68.5 bits), Expect = 1.1e-30, Sum P(3) = 1.1e-30
                  Identities = 33/71 (46%), Positives = 43/71 (60%), Frame = +1

   Query:     43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                 + + NIR+LR  ARE   E L  M EKL VV+ ERRE+    AAE  ER RK Q+  +++
   Sbjct:      5 KILNNIRTLRAQARECTLETLERMLEKLEVVVNERREEESAAAAEVEERTRKIQQYREML 64

   Query:    223 AGEGFSPEELL 255
                 +G  P ELL
   Sbjct:     65 IADGIDPNELL 75

>sp|P18955|HNS_SERMA DNA-BINDING PROTEIN H-NS (HISTONE-LIKE PROTEIN HLP-II).
>pir||S02775 DNA-binding protein H-NS - Serratia marcescens Length = 135
Plus Strand HSPs: Score = 151 (69.5 bits), Expect = 1.4e-29, Sum P(3) = 1.4e-29
                  Identities = 33/71 (46%), Positives = 44/71 (61%), Frame = +1

   Query:     43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                 + + NIR+LR  ARE   E L EM EKL VV+ ERRE+  + AE  ER RK Q+  +++
   Sbjct:      6 KILNNIRTLRAQARECTLETLEEMLEKLEVVVNERREEDSQAQAEIEERTRKLQQYREML 65

   Query:    223 AGEGFSPEELL 255
                 +G  P ELL
   Sbjct:     66 IADGIDPNELL 76

>sp|P18818|HNS_PROVU DNA-BINDING PROTEIN H-NS (HISTONE-LIKE PROTEIN HLP-II).
>pir||S02776 DNA-binding protein H-NS - Proteus vulgaris Length = 134
Plus Strand HSPs: Score = 157 (72.2 bits), Expect = 7.2e-28, Sum P(3) = 7.2e-28
                  Identities = 35/88 (39%), Positives = 51/88 (57%), Frame = +1

   Query:     43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                 + + NIR+LR  ARE   E L EM EKL VV+ ERRE+  + AE  ER++K Q+  +L+
   Sbjct:      6 KILNNIRTLRAQARETSLETLEEMLEKLEVVVNERREEEQAMQAEIEERQQKLQKYRELL 65

   Query:    223 AGEGFSPEELLGLSEEAPKSRKKTLPKP 306
                 +G  P +LL  +  +   R K   +P
   Sbjct:     66 IADGIDPTDLLEAAGASKTGRAKRAARP 93

>gi|154220 (M37891) histone H1-like protein (H-NS) [Salmonella typhimurium] Length = 137
Plus Strand HSPs: Score = 157 (72.2 bits), Expect = 1.5e-24, Sum P(2) = 1.5e-24
                  Identities = 36/82 (43%), Positives = 46/82 (56%), Frame = +1

   Query:     43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                 + + NIR+LR  ARE   E L EM EKL VV+ ERRE+    AAE  ER RK Q+  +++
   Sbjct:      6 KILNNIRTLRAQARECTLETLEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYREML 65

   Query:    223 AGEGFSPEELLGLSEEAPKSRK 288
                 +G  P ELL     A    K
   Sbjct:     66 IADGIDPNELLNSMAAAKSGTK 87

>sp|P17428|HNS_SALTY DNA-BINDING PROTEIN H-NS (HISTONE-LIKE PROTEIN HLP-II)
(PROTEIN H1) (PROTEIN B1). >pir||S10155 DNA-binding protein H-NS -
Salmonella typhimurium.>gi|47736 (X14375) H-NS protein (AA 1-137)
[Salmonella typhimurium] Length = 137
Plus Strand HSPs: Score = 157 (72.2 bits), Expect = 1.5e-24, Sum P(2) = 1.5e-24
                  Identities = 36/82 (43%), Positives = 46/82 (56%), Frame = +1

   Query:     43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
                 + + NIR+LR  ARE   E L EM EKL VV+ ERRE+    AAE  ER RK Q+  +++
   Sbjct:      6 KILNNIRTLRAQARECTLETLEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYREML 65

   Query:    223 AGEGFSPEELLGLSEEAPKSRK 288
                 +G  P ELL     A    K
   Sbjct:     66 IADGIDPNELLNSMAAAKSGTK 87
```

```
>sp|P43841|HNS_HAEIN DNA-BINDING PROTEIN H-NS HOMOLOG. >pir||D64131 DNA-binding
protein H-NS (hns) homolog - Haemophilus influenzae (strain Rd KW20)
>gi|1007825 (L46217) DNA-binding protein H-NS [Haemophilus influenzae]
>gi|1205819 (U00085) DNA-binding protein H-NS [Haemophilus influenzae]
>gi|1221730 (U32779) DNA-binding protein [Haemophilus influenzae] Length = 134.
Plus Strand HSPs: Score = 114 (52.4 bits), Expect = 1.2e-21, Sum P(2) = 1.2e-21
                  Identities = 26/70 (37%), Positives = 37/70 (52%), Frame = +1

   Query:    43 RTMMNIRSLRVFAREIDFEQLLEMQEKLNVVIEERREDAEREAAERAERERKRQELLQLI 222
               R + N+RSLR   RE+  EQ     EKL   IEE+R +         ER+ +  +  +L+
   Sbjct:     6 RGLTNLRSLRAAVRELTLEQAENALEKLQTAIEEKRANEAELIKAETERKERLAKYKELM 65

   Query:   223 AGEGFSPEEL 252
                EG +PEEL
   Sbjct:    66 EKEGITPEEL 75

>gi|47036 (X13131) kcpA gene product (AA 1 - 109) [Shigella flexneri] Length = 109
Plus Strand HSPs: Score = 110 (50.6 bits), Expect = 1.9e-21, Sum P(3) = 1.9e-21
                  Identities = 23/48 (47%), Positives = 30/48 (62%), Frame = +1

   Query:   112 MQEKLNVVIEERREDAEREAAERAERERKRQELLQLIAGEGFSPEELL 255
               M EKL VV+ ERRE+    AAE  ER RK Q+  +++  +G  P ELL
   Sbjct:     1 MLEKLEVVVNERREEESAAAAEVEERTRKLQQYREMLIADGIDPNELL 48
```

2. MtfI.

```
Sequences producing High-scoring Segment Pairs:      Reading  High   Probability
                                                     Frame    Score  P(N)      N
   gi|1197457           (U47048) microcin 24 immunity pro... +1     495   2.7e-63   1
   sp|P24884|NU5M_ASCSU NADH-UBIQUINONE OXIDOREDUCTASE CH... +3      42   1.5e-05   4
   pir||B26696          hypothetical protein 1 (CYb-COII ... +1      52   2.0e-05   3
   pir||A35349          H+-transporting ATP synthase (EC ... +1      55   0.0015    3
   gi|1109903           (U41546) coded for by C. elegans ... +2      37   0.0017    4
   pir||S43955          CR5 protein - Trypanosoma brucei ... +3      44   0.016     3
   gi|1022918           (U38184) ATPase subunit 6 [Trypan... +1      55   0.021     2
   sp|P24499|ATP6_TRYBB ATP SYNTHASE A CHAIN (PROTEIN 6).    +1      46   0.28      3
   gi|343544            (M33228) ATPase 6 [Trypanosoma br... +1      46   0.29      3
   gi|786217            (M68929) ORF238 [Marchantia polym... +2      41   0.31      3
   sp|P38459|YM16_MARPO HYPOTHETICAL 29.4 KD PROTEIN IN N... +2      41   0.33      3
   gi|992700            (U33447) G-protein-coupled recept... +1      57   0.41      2
   pir||S34960          NADH dehydrogenase (ubiquinone) (... +1      51   0.45      3
   gi|501027            (U01849) ORF2 [Trypanosoma brucei]   +3      54   0.47      2
   pir||E22845          hypothetical protein 4 - Trypanos... +1      49   0.78      2
   gi|662891            (Z48045) C41C4.7 [Caenorhabditis ... +1      42   0.80      3
   pir||A27507          DNA adenine methylase (EC 2.1.1.-... -1      44   0.95      3
   pir||S55409          cymG protein - Klebsiella oxytoca... +1      37   0.95      3
   sp|P09045|NU1M_LOCMI NADH-UBIQUINONE OXIDOREDUCTASE CH... +1      44   0.98      3
   pir||C30010          hypothetical ORF-6 protein - Saur... +1      44   0.992     3
   gi|758171            (Z48930) succinate dehydrogenase ... +1      46   0.992     2
   gi|872064            (Z49909) C14A4.10 [Caenorhabditis... +2      41   0.992     3
   pir||B21124          Bkm-like sex-determining region h... +1      62   0.995     1
   gi|436923            (U01849) ORF1 [Trypanosoma brucei]   +1      52   0.997     2
   sp|P39109|YCFI_YEAST METAL RESISTANCE PROTEIN YCF1 (YE... +2      46   0.998     3
   pir||S51863          cadmium resistance protein - yeas... +2      46   0.998     3
   gi|336843            (M57911) NADH dehydrogenase subun... +1      39   0.998     3
   pat|US|5429921|7     Sequence 7 from patent US 5429921    +1      45   0.998     3
   sp|P15583|NU5M_LEITA NADH-UBIQUINONE OXIDOREDUCTASE CH... +1      61   0.9993    1
   pir||S51910          G4 protein - Sauroleishmania tare... +1      47   0.9998    2
   gi|1171589           (X95275) frameshift [Plasmodium f... +3      42   0.9998    3

>gi|1197457 (U47048) microcin 24 immunity protein [Escherichia coli] Length = 93
Plus Strand HSPs: Score = 495 (227.7 bits), Expect = 2.7e-63, P = 2.7e-63
     Identities = 93/93 (100%), Positives = 93/93 (100%), Frame = +1

   Query:     1 MSFLNFAFSPVFFSIMACYFIVWRNKRNEFVCNRLLSIIIISFLICFIYPWLNYKIEVKY 180
                MSFLNFAFSPVFFSIMACYFIVWRNKRNEFVCNRLLSIIIISFLICFIYPWLNYKIEVKY
   Sbjct:     1 MSFLNFAFSPVFFSIMACYFIVWRNKRNEFVCNRLLSIIIISFLICFIYPWLNYKIEVKY 60

   Query:   181 YIFEQFYLFCFLSSLVAVVINLIVYFILYRRCI 279
                YIFEQFYLFCFLSSLVAVVINLIVYFILYRRCI
   Sbjct:    61 YIFEQFYLFCFLSSLVAVVINLIVYFILYRRCI 93
```

```
>sp|P24884|NU5M_ASCSU NADH-UBIQUINONE OXIDOREDUCTASE CHAIN 5.

Score = 41 (18.9 bits), Expect = 1.5e-05, Sum P(4) = 1.5e-05
Identities = 8/19 (42%), Positives = 12/19 (63%), Frame = +1

   Query:   181 YIFEQFYLFCFLSSLVAVV 237
               Y F     FC+LSSL+ ++
   Sbjct:   155 YYFLSLSFFCWLSSLMLLL 173

Score = 40 (18.4 bits), Expect = 6.9, Sum P(3) = 1.0
Identities = 8/17 (47%), Positives = 9/17 (52%), Frame = +1

   Query:    10 LNFAFSPVFFSIMACYF 60
               L F FS  F  + AC F
   Sbjct:   350 LEFFFSNFFMVVFACMF 366

Score = 38 (17.5 bits), Expect = 1.5e-05, Sum P(4) = 1.5e-05
Identities = 8/17 (47%), Positives = 12/17 (70%), Frame = +1

   Query:   229 AVVINLIVYFILYRRCI 279
               +VVI L+++F L   CI
   Sbjct:   516 SVVIVLVLFFFLVWGCI 532

Score = 34 (15.6 bits), Expect = 0.00033, Sum P(4) = 0.00033
Identities = 6/11 (54%), Positives = 8/11 (72%), Frame = +1

   Query:   196 FYLFCFLSSLV 228
               F+LF F SS +
   Sbjct:   141 FFLFVFFSSTI 151

Score = 34 (15.6 bits), Expect = 3.0, Sum P(3) = 0.95
Identities = 7/19 (36%), Positives = 12/19 (63%), Frame = +1

   Query:    91 VCNRLLSIIIISFLICFIY 147
               V    LS++++ F I FI+
   Sbjct:   399 VVMNFLSLLLVLFSIFFIW 417

Score = 33 (15.2 bits), Expect = 1.5e-05, Sum P(4) = 1.5e-05
Identities = 8/14 (57%), Positives = 9/14 (64%), Frame = +1

   Query:     7 FLNFAFSPVFFSIM 48
               FL+F  S  F SIM
   Sbjct:    37 FLSFKISVYFNSIM 50

Score = 32 (14.7 bits), Expect = 1.5e-05, Sum P(4) = 1.5e-05
Identities = 6/29 (20%), Positives = 16/29 (55%), Frame = +1

   Query:   103 LLSIIIISFLICFIYPWLNYKIEVKYYIF 189
               L+ +++   ++ F   +L+ ++   YY F
   Sbjct:    53 LILLLVTISVLVFSTYYLSGELNFNYYYF 81

>pir||B26696 hypothetical protein 1 (CYb-COII intergenic region) - Sauroleishmania
tarentolae mitochondrion (SGC6) (fragment)
>gi|896286 (M10126) NH2 terminus uncertain [Sauroleishmania tarentolae] Length = 443
Plus Strand HSPs: Score = 52 (23.9 bits), Expect = 2.0e-05, Sum P(3) = 2.0e-05
                  Identities = 10/33 (30%), Positives = 18/33 (54%), Frame = +1

   Query:   166 IEVKYYIFEQFYLFCFLSSLVAVVINLIVYFIL 264
               I  K YIF  +  F  + SL  +++ ++ YF +
   Sbjct:   276 ISTKNYIFYMYLNFHLIYSLSLIILIILYYFFI 308

Score = 45 (20.7 bits), Expect = 2.0e-05, Sum P(3) = 2.0e-05
Identities = 9/15 (60%), Positives = 10/15 (66%), Frame = +1

   Query:   103 LLSIIIISFLICFIY 147
               L   I+I  LICFIY
   Sbjct:   216 LFCFILIILLICFIY 230

Score = 37 (17.0 bits), Expect = 2.0e-05, Sum P(3) = 2.0e-05
Identities = 9/21 (42%), Positives = 12/21 (57%), Frame = +1

   Query:     7 FLNFAFSPVFFSIMACYFIVW 69
               FLNF    VF  I+  Y I++
   Sbjct:    71 FLNFDTRFVFMIIIMQYIIIF 91
```

```
Score = 33 (15.2 bits), Expect = 1.3, Sum P(3) = 0.73
Identities = 6/26 (23%), Positives = 15/26 (57%), Frame = +1

   Query:   190 EQFYLFCFLSSLVAVVINLIVYFILY 267
               + +  + +L+  +   ++LI+  ILY
   Sbjct:   279 KNYIFYMYLNFHLIYSLSLIILIILY 304

>pir||A35349 H+-transporting ATP synthase (EC 3.6.1.34) protein 6 - Trypanosoma brucei
mitochondrion (SGC6) Length = 214
Plus Strand HSPs: Score = 55 (25.3 bits), Expect = 0.0015, Sum P(3) = 0.0015
                  Identities = 10/29 (34%), Positives = 18/29 (62%), Frame = +1

   Query:   178 YYIFEQFYLFCFLSSLVAVVINLIVYFIL 264
               Y+IF  F+L+CFL  +  +   ++  FI+
   Sbjct:   166 YFIFVFFFLWCFLLLIYFIYFCVLFLFII 194

Score = 36 (16.6 bits), Expect = 0.0015, Sum P(3) = 0.0015
Identities = 8/38 (21%), Positives = 17/38 (44%), Frame = +1

   Query:    31 VFFSIMACYFIVWRNKRNEFVCNRLLSIIIISFLICFI 144
               + F  + C+   +    N  V +  + +  I  L+CF+
   Sbjct:    94 IAFLFLFCFLCDFFLFNNLLVGDSFMDVFFIRPLLCFL 131

Score = 32 (14.7 bits), Expect = 0.011, Sum P(3) = 0.011
Identities = 7/17 (41%), Positives = 8/17 (47%), Frame = +1

   Query:    94 CNRLLSIIIISFLICFI 144
               C   L  I   FL CF+
   Sbjct:    87 CIVFLLYIAFLFLFCFL 103
```

3. MtfS.

```
Sequences producing High-scoring Segment Pairs:      Reading High  Probability
                                                     Frame Score  P(N)      N

   gi|1197458           (U47048) microcin 24 precursor [E... +1   394  2.7e-49   1
   sp|P04052|RPB1_DROME DNA-DIRECTED RNA POLYMERASE II LA... +2    37  0.0046    4
   pir||S24066          protein-tyrosine kinase (EC 2.7.1... +1    44  0.0083    3
   sp|P35590|TIE1_HUMAN TYROSINE-PROTEIN KINASE RECEPTOR ... +1    44  0.0083    3
   sp|Q06805|TIE1_BOVIN TYROSINE-PROTEIN KINASE RECEPTOR ... +1    44  0.015     3
   pir||A36734          bacillopeptidase F (EC 3.4.21.-) ... +1    46  0.030     3
   gi|1139531           (D44498) 90k-protease (bacillopep... +1    46  0.030     3
   sp|P36329|POLS_EEVV3 STRUCTURAL POLYPROTEIN (CONTAINS:... -1    42  0.032     3
   gi|554636            (L00930) polyprotein [Venezuelan ... -1    42  0.034     3
   sp|P09592|POLS_EEVVT STRUCTURAL POLYPROTEIN (CONTAINS:... -1    42  0.056     3
   pir||VHWVVT          structural polyprotein - Venezuel... -1    42  0.056     3
   gi|995557            (X89453) pid:e188462 [Rattus norv... -2    37  0.067     3
   sp|P42345|FRAP_HUMAN FKBP-RAPAMYCIN ASSOCIATED PROTEIN... +2    38  0.085     3
   sp|P42346|FRAP_RAT   FKBP-RAPAMYCIN ASSOCIATED PROTEIN... +2    38  0.085     3
   pir||S45340          FKBP-rapamycin-associated protein... +2    38  0.085     3
   prf||2014422A        FKBP-rapamycin-associated protein... +2    38  0.085     3
   pir||S37671          bat2 protein - human >gi|29375 (Z... -2    41  0.15      3
   pir||S36152          bat2 protein - human                -2    41  0.15      3
   pir||S41525          major ring-forming surface protei... +1    36  0.16      3
   sp|P05674|POLS_EEVV8 STRUCTURAL POLYPROTEIN (CONTAINS:... -1    42  0.27      3
   sp|P36332|POLS_EEVVP STRUCTURAL POLYPROTEIN (CONTAINS:... -1    42  0.27      3
   pir||A38096          heparan sulfate proteoglycan HSPG... +1    40  0.41      3
   pir||A41736          heparan sulfate proteoglycan prec... +1    40  0.41      3
   gi|29470             (X62515) Human basement membrane ... +1    40  0.41      3
   pir||A41793          dipeptidyl aminopeptidase-like pr... +1    37  0.61      3
   gi|306708            (M96860) dipeptidyl aminopeptidas... +1    37  0.61      3
   sp|P42659|DPP6_BOVIN DIPEPTIDYL PEPTIDASE IV LIKE PROT... +1    37  0.66      3
   sp|P42658|DPP6_HUMAN DIPEPTIDYL PEPTIDASE IV LIKE PROT... +1    37  0.66      3
   sp|P22567|ARGA_PSEAE AMINO-ACID ACETYLTRANSFERASE (N-A... +3    36  0.70      3
   gi|993017            (X87246) alternative start codon ... -1    41  0.73      3
   gi|993016            (X87246) alternative start codon ... -1    41  0.73      3
   pir||A27598          major surface antigen precursor -... +1    36  0.82      3
   sp|P08148|GP63_LEIMA LEISHMANOLYSIN PRECURSOR (CELL SU... +1    36  0.82      3
   sp|P10264|GAG2_HUMAN RETROVIRUS-RELATED GAG POLYPROTEI... +1    46  0.83      2
   gi|1196427           (M14123) gag 2 protein [Homo sapi... +1    46  0.85      2
   gi|190164            (M73548) polyposis locus-encoded ... -1    47  0.85      2
   sp|P25054|APC_HUMAN  ADENOMATOUS POLYPOSIS COLI PROTEI... -1    47  0.86      2
   pir||B39658          polyposis coli protein DP2.5 - hu... -1    47  0.86      2
   gi|887448            (X72791) pid:e185416 [Human endog... +1    46  0.95      2
   pir||S55202          hypothetical protein YJR83.24 - y... -1    62  0.97      1
   gi|300249            (S60312) DMR-N9 {C-terminal} [mic... -1    48  0.97      2
   gi|1049102           (U31777) atrophin-1 [Rattus norve... -2    37  0.97      3
   gi|644882            (D45419) HCF [Mesocricetus auratus]  +1    39  0.98      3
   gi|817954            (Z38011) pid:g817954 [Mus musculus]  -1    48  0.98      2
```

```
   sp|P03186|TEGU_EBV   LARGE TEGUMENT PROTEIN. >pir||QQB... +3    39  0.996     3
   gi|290623            (L04599) envelope glycoprotein [V... -1    38  0.999     3
   gi|291946            (L12398) dopamine receptor D4 [Ho... +3    36  0.999     3
   gi|191992            (M88127) APC [Mus musculus]          -1    47  0.9993    2
   gi|915326            (U23851) atrophin-1 [Homo sapiens]   -3    35  0.9998    3
   pir||S50832          DRPLA protein - human                -3    35  0.9998    3
   gi|862330            (D31840) DRPLA gene product [Homo... -3    35  0.9998    3
   pir||A53689          transcription factor ITF2 - rat (... +1    45  0.9998    2
   pir||S41735          cholesterol esterase - yeast (Can... -2    36  0.9999    3
   gi|157018            (L06475) brain-specific homeobox ... +1    60  0.9999    1
   sp|P35914|HMGL_HUMAN HYDROXYMETHYLGLUTARYL-COA LYASE P... +1    49  0.99990   2
   sp|P32947|LIP3_CANRU LIPASE 3 PRECURSOR. >pir||JN0551 ... -2    36  0.99993   3
   gi|1212992           (X90568) Protein sequence and ann... +1    42  0.99994   3

>gi|1197458 (U47048) microcin 24 precursor [Escherichia coli] Length = 90
Plus Strand HSPs: Score = 394 (181.2 bits), Expect = 2.7e-49, P = 2.7e-49
                  Identities = 74/74 (100%), Positives = 74/74 (100%), Frame = +1

   Query:     1 AGDPLADPNSQIVRQIMSNAAWGPPLVPERFRGMAVGAAGGVTQTVLQGAAAHMPVNVPI 180
                AGDPLADPNSQIVRQIMSNAAWGPPLVPERFRGMAVGAAGGVTQTVLQGAAAHMPVNVPI
   Sbjct:    17 AGDPLADPNSQIVRQIMSNAAWGPPLVPERFRGMAVGAAGGVTQTVLQGAAAHMPVNVPI 76

   Query:   181 PKVPMGPSWNGSKG 222
               PKVPMGPSWNGSKG
   Sbjct:    77 PKVPMGPSWNGSKG 90

>sp|P04052|RPB1_DROME DNA-DIRECTED RNA POLYMERASE II LARGEST SUBUNIT.
>pir||RNFF2L DNA-directed RNA polymerase (EC 2.7.7.6) II 215K chain
 - fruit fly (Drosophila melanogaster) >gi|158332 (M27431) RNA polymerase II [Drosophila
melanogaster] Length = 1896
Plus Strand HSPs:
Score = 34 (15.6 bits), Expect = 0.0046, Sum P(4) = 0.0046
Identities = 6/11 (54%), Positives = 8/11 (72%), Frame = +1

   Query:   190 PMGPSWNGSKG 222
               P  PS++GS G
   Sbjct:  1765 PPSPSYDGSPG 1775

Score = 31 (14.3 bits), Expect = 0.0046, Sum P(4) = 0.0046
Identities = 5/9 (55%), Positives = 7/9 (77%), Frame = +1

   Query:     4 GDPLADPNS 30
               GD +ADP +
   Sbjct:   682 GDTIADPQT 690

>pir||S24066 protein-tyrosine kinase (EC 2.7.1.112) Tie - human >gi|512414 (A16753) tie
receptor tyrosine kinase gene product [Homo sapiens]
>pat|US|5447860|8 Sequence 8 from patent US 5447860 Length = 1138
Plus Strand HSPs: Score = 44 (20.2 bits), Expect = 0.0083, Sum P(3) = 0.0083
      Identities = 7/10 (70%), Positives = 9/10 (90%), Frame = +1

   Query:    61 AWGPPLVPER 90
               AWGPPL+ E+
   Sbjct:    56 AWGPPLLLEK 65

Score = 35 (16.1 bits), Expect = 0.0083, Sum P(3) = 0.0083
Identities = 6/10 (60%), Positives = 8/10 (80%), Frame = +1

   Query:   166 VNVPIPKVPM 195
               VNV +P VP+
   Sbjct:   441 VNVKVPPVPL 450

>sp|P35590|TIE1_HUMAN TYROSINE-PROTEIN KINASE RECEPTOR TIE-1 PRECURSOR.
>gi|396815 (X60957) receptor tyrosine kinase [Homo sapiens] Length = 1138
Plus Strand HSPs: Score = 44 (20.2 bits), Expect = 0.0083, Sum P(3) = 0.0083
      Identities = 7/10 (70%), Positives = 9/10 (90%), Frame = +1

   Query:    61 AWGPPLVPER 90
               AWGPPL+ E+
   Sbjct:    56 AWGPPLLLEK 65

Score = 35 (16.1 bits), Expect = 0.0083, Sum P(3) = 0.0083
identities = 6/10 (60%), Positives = 8/10 (80%), Frame = +1

   Query:   166 VNVPIPKVPM 195
               VNV +P VP+
   Sbjct:   441 VNVKVPPVPL 450
```

```
>sp|Q06805|TIE1_BOVIN TYROSINE-PROTEIN KINASE RECEPTOR TIE-1 PRECURSOR.
>pir||S32690 protein-tyrosine kinase (EC 2.7.1.112) Tie-1 precursor - bovine
>gi|296576 (X71423) receptor tyrosine kinase [Bos taurus] Length = 1136
Plus Strand HSPs: Score = 44 (20.2 bits), Expect = 0.015, Sum P(3) = 0.015
                  Identities = 7/10 (70%), Positives = 9/10 (90%), Frame = +1

   Query:    61 AWGPPLVPER 90
               AWGPPL+ E+
   Sbjct:    56 AWGPPLLLEK 65

Score = 34 (15.6 bits), Expect = 0.015, Sum P(3) = 0.015
Identities = 5/10 (50%), Positives = 8/10 (80%), Frame = +1

   Query:   166 VNVPIPKVPM 195
               +NV +P VP+
   Sbjct:   439 INVKVPPVPL 448

>pir||A36734 bacillopeptidase F (EC 3.4.21.-) precursor - Bacillus subtilis
>gi|143308 (M29035) bacillopeptidase F [Bacillus subtilis] Length = 1433
Plus Strand HSPs: Score = 46 (21.2 bits), Expect = 0.031, Sum P(3) = 0.030
      Identities = 8/27 (29%), Positives = 13/27 (48%), Frame = +1

   Query:    19 DPNSQIVRQIMSNAAWGPPLVPERFRG 99
               D    +V  I +   W  P + E++RG
   Sbjct:   217 DGTGTVVASIDTGVEWNHPALKEKYRG 243

>gi|1139531 (D44498) 90k-protease (bacillopeptidase F) [Bacillus subtilis] Length = 1433
Plus Strand HSPs:       Score = 46 (21.2 bits), Expect = 0.031, Sum P(3) = 0.030
                  Identities = 8/27 (29%), Positives = 13/27 (48%), Frame = +1

   Query:    19 DPNSQIVRQIMSNAAWGPPLVPERFRG 99
               D    +V  I +   W  P + E++RG
   Sbjct:   217 DGTGTVVASIDTGVEWNHPALKEKYRG 243

>sp|P42345|FRAP_HUMAN FKBP-RAPAMYCIN ASSOCIATED PROTEIN (FRAP) (RAPAMYCIN TARGET
PROTEIN). >gi|508482 (L34075) FKBP-rapamycin associated protein [Homo sapiens] Length =
2549
Plus Strand HSPs:
Score = 36 (16.6 bits), Expect = 0.089, Sum P(3) = 0.085
Identities = 5/16 (31%), Positives = 10/16 (62%), Frame = +1

   Query:     7 DPLADPNSQIVRQIMS 54
               DP  DPN  ++  +++
   Sbjct:   785 DPDPDPNPGVINNVLA 800

>sp|P42346|FRAP_RAT FKBP-RAPAMYCIN ASSOCIATED PROTEIN (FRAP) (RAPAMYCIN TARGET PROTEIN).
>pir||A54837 rapamycin/FKBP12 target 1 - rat
>gi|511229 (U11681) rapamycin and FKBP12 target-1 protein [Rattus norvegicus]
>gi|561858 (L37085) rapamycin target [Rattus norvegicus]Length = 2549
Plus Strand HSPs:
Score = 36 (16.6 bits), Expect = 0.089, Sum P(3) = 0.085
Identities = 5/16 (31%), Positives = 10/16 (62%), Frame = +1

>pir||S45340 FKBP-rapamycin-associated protein (FRAP) - human Length = 2549
Plus Strand HSPs:
Score = 36 (16.6 bits), Expect = 0.089, Sum P(3) = 0.085
Identities = 5/16 (31%), Positives = 10/16 (62%), Frame = +1

   Query:     7 DPLADPNSQIVRQIMS 54
               DP  DPN  ++  +++
   Sbjct:   785 DPDPDPNPGVINNVLA 800

>prf||2014422A FKBP-rapamycin-associated protein [Homo sapiens] Length = 2549
Plus Strand HSPs:
Score = 36 (16.6 bits), Expect = 0.089, Sum P(3) = 0.085
Identities = 5/16 (31%), Positives = 10/16 (62%), Frame = +1

   Query:     7 DPLADPNSQIVRQIMS 54
               DP  DPN  ++  +++
   Sbjct:   785 DPDPDPNPGVINNVLA 800
```

5. MtfA

```
Sequences producing High-scoring Segment Pairs:      Reading High  Probability
                  Frame Score  P(N)       N

   gi|1197459           (U47048) MtfA [Escherichia coli]     +1  2120  1.1e-287  1
   sp|P22519|CVAA_ECOLI COLICIN V SECRETION PROTEIN CVAA.... +1  1599  7.3e-216  1
   sp|P09986|HLY4_ECOLI HEMOLYSIN D, CHROMOSOMAL. >pir||L... +1    97  1.4e-10   4
   sp|P06739|HLYD_ECOLI HEMOLYSIN D, PLASMID. >pir||S1005... +1    87  3.3e-09   4
   sp|P18790|LKTD_ACTAC LKTD PROTEIN. >pir||S11215 lktD p... +1    81  1.9e-07   4
   gnl|PID|e152894      (X86087) EHEC-hlyD gene product [... +1    64  4.8e-05   4
   sp|P16534|LKTD_PASHA LKTD PROTEIN. >pir||B32051 leukot... +1    84  0.00024   3
   gi|150515            (M24197) leukotoxin (LktD) [Paste... +1    84  0.00024   3
   pir||D30169          hypothetical 54.7K protein - Past... +1    84  0.00024   3
   sp|P44812|YIIU_HAEIN HYPOTHETICAL PROTEIN HI0668. >pir... +1    58  0.00061   2
   gi|141822            (M65808) hemolysin determinant [A... +1    71  0.022     3
   pir||S18856          *ClyI-D protein - Actinobacillus ... +1    71  0.022     3
   pir||S48045          xIIID protein - Actinobacillus pl... +1    70  0.023     2
   sp|Q08633|RTXD_ACTPL RTX TOXIN-III OPERON PROTEIN RTXD... +1    69  0.032     2
   sp|P26761|HLYD_ACTPL HEMOLYSIN SECRETION PROTEIN APPD ... +1    62  0.033     4
   gi|805105            (U22021) P120 [Mycoplasma hominis]   +1    71  0.27      2
   gi|457620            (L12148) pllktD [Pasteurella haem... +1    75  0.41      2
   gi|1208411           (D38539) CeTMIII [Caenorhabditis ... +1    77  0.43      1
   sp|Q09712|YA35_SCHPO HYPOTHETICAL 48.8 KD PROTEIN C18B... -1    59  0.52      2
   gi|298680            (S57152) placenta growth factor 2... +3    59  0.84      2
   gi|633814            (S72960) placenta growth factor 2... +3    59  0.84      2
   gi|1139568           (D49826) Serratia marcescens LipB... +1    59  0.89      3
   gi|695621            (Z48542) SppE [Lactobacillus sake]   +1    61  0.91      3
   gi|733567            (U23452) No definition line found... +1    52  0.92      4
   gi|1046031           (U39715) protein V [Mycoplasma ge... +1    73  0.92      1
   gi|1150766           (U42409) myosin heavy chain [Dict... +1    75  0.93      2
   sp|P32716|YJCR_ECOLI HYPOTHETICAL 36.9 KD PROTEIN IN F... +1    64  0.94      2
   gi|599857            (Z46867) accessory factor for ABC... +1    70  0.96      2
   gi|1212983           (X95654) polypeptide of 976 aa [H... +1    48  0.96      4
   gi|806511            (D50474) fast skeletal muscle myo... +1    74  0.97      2
   gi|694116            (U21056) polyprotein [Yellow feve... +1    59  0.98      3
   gi|829367            (U17066) polyprotein precursor [Y... +1    59  0.98      3
   gi|1065509           (U40419) C27F2.6 gene product [Ca... -3    54  0.98      3
   sp|Q03030|DCOA_SALTY OXALOACETATE DECARBOXYLASE ALPHA ... +1    58  0.99      2
   gi|805101            (U22019) P120 [Mycoplasma hominis]   +1    64  0.99      2
   sp|Q02224|CENE_HUMAN CENTROMERIC PROTEIN E (CENP-E PRO... +1    58  0.997     2
   prf||1819485A        CENP-E protein [Homo sapiens]        +1    58  0.997     2
   gi|806513            (D50475) fast skeletal muscle myo... +1    74  0.998     2
   pir||S54487          Bullp protein (YM8156.17c) - yeas... +1    56  0.9992    2
   gi|773190            (D50083) the BUL1 gene product [S... +1    56  0.9993    2
   gi|895907            (X88901) respiration deficiency s... +1    56  0.9993    2
   gi|1177653           (X92557) biotin carboxylase and b... +1    63  0.9996    2
   sp|P09490|TPM0_DROME TROPOMYOSIN I, MUSCLE EMBRYONIC I... +1    69  0.9996    1
   pir||S05515          myosin heavy chain, skeletal musc... +1    69  0.9998    1
   sp|P03314|POLG_YEFV1 GENOME POLYPROTEIN (CONTAINS: CAP... +1    57  0.9998    3
   gi|694114            (U21055) polyprotein [Yellow feve... +1    57  0.9998    3
   pir||C35815          myosin heavy chain 3, muscle - fr... +1    56  0.9998    2
   pir||D35815          myosin heavy chain 4, muscle - fr... +1    56  0.9998    2
   sp|P11154|PYC1_YEAST PYRUVATE CARBOXYLASE 1 (PYRUVIC C... +1    62  0.9999    2
   pir||A35815          myosin heavy chain 1, muscle - fr... +1    56  0.9999    2
   pir||B35815          myosin heavy chain 2, muscle - fr... +1    56  0.9999    2
   gi|1208409           (D38539) CeTMI [Caenorhabditis el... +1    58  0.99995   2
   gi|1208413           (D38540) CeTMI [Caenorhabditis el... +1    58  0.99995   2


>gi|1197459 (U47048) MtfA [Escherichia coli] Length = 414
Plus Strand HSPs: Score = 2120 (971.0 bits), Expect = 1.1e-287, P = 1.1e-287
                  Identities = 414/414 (100%), Positives = 414/414 (100%), Frame = +1

   Query:     1 MKWCGRAVLLPGIPPWLVISVSVFFFISFIVFVMEGTYTRRINVEGEITTWPRPVNVYSG 180
                MKWCGRAVLLPGIPPWLVISVSVFFFISFIVFVMEGTYTRRINVEGEITTWPRPVNVYSG
   Sbjct:     1 MKWCGRAVLLPGIPPWLVISVSVFFFISFIVFVMEGTYTRRINVEGEITTWPRPVNVYSG 60

   Query:   181 VQGVVIKQFVTEGQRIKKGDPIYLIDVSKSTSSGVVGDNKRRDIEKQLSRIGDIISRLEE 360
                VQGVVIKQFVTEGQRIKKGDPIYLIDVSKSTSSGVVGDNKRRDIEKQLSRIGDIISRLEE
   Sbjct:    61 VQGVVIKQFVTEGQRIKKGDPIYLIDVSKSTSSGVVGDNKRRDIEKQLSRIGDIISRLEE 120

   Query:   361 NKKTTLQTLEKQRIQYYSAFERSTEILRRAEEGVKIMKSNMDNYKQYQTKGLINKDQLTN 540
                NKKTTLQTLEKQRIQYYSAFERSTEILRRAEEGVKIMKSNMDNYKQYQTKGLINKDQLTN
   Sbjct:   121 NKKTTLQTLEKQRIQYYSAFERSTEILRRAEEGVKIMKSNMDNYKQYQTKGLINKDQLTN 180

   Query:   541 QIALYYQQQNNILSLSTQNEQNLLQVTSLESQMQTLAAEFDNRIYQVELQRYELQKELVD 720
                QIALYYQQQNNILSLSTQNEQNLLQVTSLESQMQTLAAEFDNRIYQVELQRYELQKELVD
   Sbjct:   181 QIALYYQQQNNILSLSTQNEQNLLQVTSLESQMQTLAAEFDNRIYQVELQRYELQKELVD 240
```

```
   Query:   721 TDAGRDIIIRALSDGKIDSLSVTPGQMVSVGDSLLQIIPEEIKNYHLIVWVPNNAIPYIS 900
                TDAGRDIIIRALSDGKIDSLSVTPGQMVSVGDSLLQIIPEEIKNYHLIVWVPNNAIPYIS

   Sbjct:   241 TDAGRDIIIRALSDGKIDSLSVTPGQMVSVGDSLLQIIPEEIKNYHLIVWVPNNAIPYIS 300

   Query:   901 VGDNVNVRYEAFPPGKFGQFTAKIMLISRTPASAQEMQTYPGAPRNNTGVSVPYYKIVLN 1080
                VGDNVNVRYEAFPPGKFGQFTAKIMLISRTPASAQEMQTYPGAPRNNTGVSVPYYKIVLN
   Sbjct:   301 VGDNVNVRYEAFPPGKFGQFTAKIMLISRTPASAQEMQTYPGAPRNNTGVSVPYYKIVLN 360

   Query:  1081 PEQQTIEYGEKKMPLENGMKAQSTLFLEKRKIYQWMLSPFYNMKYSAVGPVNEQ 1242
                PEQQTIEYGEKKMPLENGMKAQSTLFLEKRKIYQWMLSPFYNMKYSAVGPVNEQ
   Sbjct:   361 PEQQTIEYGEKKMPLENGMKAQSTLFLEKRKIYQWMLSPFYNMKYSAVGPVNEQ 414

>sp|P22519|CVAA_ECOLI COLICIN V SECRETION PROTEIN CVAA. >pir||IKEC5A colicin V
secretion protein cvaA - Escherichia coli plasmid ColV >gi|41175
(X57524) cvaA protein[Escherichia coli] Length = 413
Plus Strand HSPs: Score = 1599 (732.4 bits), Expect = 7.3e-216, P = 7.3e-216
                  Identities = 294/413 (71%), Positives = 358/413 (86%), Frame = +1

   Query:     1 MKWCGRAVLLPGIPPWLVISVSVFFFISFIVFVMEGTYTRRINVEGEITTWPRPVNVYSG 180
                MKW GRA+LLPGIP WL++  S+ F  +F++F++ GTY+RR+NV GE+TTWPR VN+YSG
   Sbjct:     1 MKWQGRAILLPGIPLWLIMLGSIVFITAFLMFIIVGTYSRRVNVSGEVTTWPRAVNIYSG 60

   Query:   181 VQGVVIKQFVTEGQRIKKGDPIYLIDVSKSTSSGVVGDNKRRDIEKQLSRIGDIISRLEE 360
                VQG V++QFV EGQ IKKGDP+YLID+SKST +G+V DN RRDIE QL R+ +IISRLEE
   Sbjct:    61 VQGFVVRQFVHEGQLIKKGDPVYLIDISKSTRNGIVTDNHRRDIENQLVRVDNIISRLEE 120

   Query:   361 NKKTTLQTLEKQRIQYYSAFERSTEILRRAEEGVKIMKSNMDNYKQYQTKGLINKDQLTN 540
                +KK TL TLEKQR+QY  AF RS++I++RAEEG+KIMK+NM+NY+ YQ+KGLINKDQLTN
   Sbjct:   121 SKKITLDTLEKQRLQYTDAFRRSSDIIQRAEEGIKIMKNNMENYRYYQSKGLINKDQLTN 180

   Query:   541 QIALYYQQQNNILSLSTQNEQNLLQVTSLESQMQTLAAEFDNRIYQVELQRYELQKELVD 720
                Q+ALYYQQQNN+LSLS QNEQN LQ+T+LESQ+QT AA+FDNRIYQ+ELQR ELQKELV+
   Sbjct:   181 QVALYYQQQNNLLSLSGQNEQNALQITTLESQIQTQAADFDNRIYQMELQRLELQKELVN 240

   Query:   721 TDAGRDIIIRALSDGKIDSLSVTPGQMVSVGDSLLQIIPEEIKNYHLIVWVPNNAIPYIS 900
                TD   +IIIRALSDGK+DSLSVT GQMV+ GDSLLQ+IPE I+NY+LI+WVPN+A+PYIS
   Sbjct:   241 TDVEGEIIIRALSDGKVDSLSVTVGQMVNTGDSLLQVIPENIENYYLILWVPNDAVPYIS 300

   Query:   901 VGDNVNVRYEAFPPGKFGQFTAKIMLISRTPASAQEMQTYPGAPRNNTGVSVPYYKIVLN 1080
                 GD VN+RYEAFP  KFGQF+A +  ISRTPAS QEM TY GAP+N  G SVP+YK++
   Sbjct:   301 AGDKVNIRYEAFPSEKFGQFSATVKTISRTPASTQEMLTYKGAPQNTPGASVPWYKVIAT 360

   Query:  1081 PEQQTIEYGEKKMPLENGMKAQSTLFLEKRKIYQWMLSPFYNMKYSAVGPVNE 1239
                PE+Q I Y EK +PLENGMKA+STLFLEKR+IYQWMLSPFY+MK+SA GP+N+
   Sbjct:   361 PEKQIIRYDEKYLPLENGMKAESTLFLEKRRIYQWMLSPFYDMKHSATGPIND 413

>sp|P09986|HLY4_ECOLI HEMOLYSIN D, CHROMOSOMAL. >pir||LEECD hemolysin secretion
protein hlyD - Escherichia coli >gi|146381 (M10133) chromosomalhemolysin D (hlyD)
[Escherichia coli] Length = 478
Plus Strand HSPs: Score = 97 (44.4 bits), Expect = 1.4e-10, Sum P(4) = 1.4e-10
                  Identities = 26/90 (28%), Positives = 40/90 (44%), Frame = +1

   Query:   742 IIRALSDGKIDSLSVTPGQMVSVGDSLLQIIPEEIKNYHLIVWVPNNAIPYISVGDNVNV 921
                +IRA   GK+  L V     V      L +I  E     +   V N  I +I+VG N  +
   Sbjct:   329 VIRAPVSGKVQQLKVHTEGGVVTTAETLMVIVPEDDTLEVTALVQNKDIGFINVGQNAII 388

   Query:   922 RYEAFPPGKFGQFTAKIMLISRTPASAQEM 1011
                + EAFP  ++G    K+  I+      Q++
   Sbjct:   389 KVEAFPYTRYGYLVGKVKNINLDAIEDQKL 418

Score = 68 (31.1 bits), Expect = 1.4e-10, Sum P(4) = 1.4e-10
Identities = 11/45 (24%), Positives = 25/45 (55%), Frame = +1

   Query:  1063 YKIVLNPEQQTIEYGEKKMPLENGMKAQSTLFLEKRKIYQWMLSP 1197
                + ++++ E+  +  G K +PL +GM   + +    R +  ++LSP
   Sbjct:   422 FNVIVSVEENDLSTGNKHIPLSSGMAVTAEIKTGMRSVISYLLSP 466

Score = 47 (21.5 bits), Expect = 1.4e-10, Sum P(4) = 1.4e-10
Identities = 15/66 (22%), Positives = 27/66 (40%), Frame = +1

   Query:    43 PWLVISVSVFFFISFIVFVMEGTYTRRINVEGEITTWPRPVNVYSGVQGVVIKQFVTEGQ 222
                P LV    + F +   +  + G          G++T   R   +        +V +  V EG+
   Sbjct:    57 PRLVAYFIMGFLVIAFILSVLGQVEIVATANGKLTLSGRSKEIKPIENSIVKEIIVKEGE 116

   Query:   223 RIKKGD 240
                 ++KGD
   Sbjct:   117 SVRKGD 122
```

```
Score = 45 (20.6 bits), Expect = 0.96, Sum P(2) = 0.62
Identities = 11/37 (29%), Positives = 20/37 (54%), Frame = +1

   Query:   721 TDAGRDIIIRALSDGKIDSLSVTPGQMVSVGDSLLQI 831
               T +GR   I+ + +  +  + V  G+ V  GD LL++
   Sbjct:    91 TLSGRSKEIKPIENSIVKEIIVKEGESVRKGDVLLKL 127

Score = 37 (16.9 bits), Expect = 1.4e-10, Sum P(4) = 1.4e-10
Identities = 9/32 (28%), Positives = 16/32 (50%), Frame = +1

   Query:   613 QVTSLESQMQTLAAEFDNRIYQVELQRYELQK 708
               +  +L++Q   L A  +   YQ+  +  EL K
   Sbjct:   133 EADTLKTQSSLLQARLEQIRYQILSRSIELNK 164

Score = 37 (16.9 bits), Expect = 1.4e-10, Sum P(4) = 1.4e-10
Identities = 9/38 (23%), Positives = 22/38 (57%), Frame = +1

   Query:   586 STQNEQNLLQVTSLESQMQTLAAEFDNRIYQVELQRYE 699
               S  ++Q + +   LE + + + A  + R+Y+ +L++ E
   Sbjct:   242 SLLHKQAIAKHAVLEQENKYVEAANELRVYKSQLEQIE 279

Score = 37 (16.9 bits), Expect = 1.4e-10, Sum P(4) = 1.4e-10
Identities = 9/38 (23%), Positives = 21/38 (55%), Frame = +1

   Query:   607 LLQVTSLESQMQTLAAEFDNRIYQVELQRYELQKELVD 720
               LL++T+L ++  TL  +      ++E  RY++    ++
   Sbjct:   124 LLKLTALGAEADTLKTQSSLLQARLEQIRYQILSRSIE 161

>sp|P06739|HLYD_ECOLI HEMOLYSIN D, PLASMID. >pir||S10058 hemolysin secretion
protein hlyD - Escherichia coli plasmid pHly152 >gi|150684 (M14107)
hemolysin D [Plasmid pHly152] >prf||1208296D hemolysin hlyD [Escherichia coli] Length =
478
Plus Strand HSPs: Score = 87 (39.8 bits), Expect = 3.3e-09, Sum P(4) = 3.3e-09
                  Identities = 24/81 (29%), Positives = 36/81 (44%), Frame = +1

   Query:   742 IIRALSDGKIDSLSVTPGQMVSVGDSLLQIIPEEIKNYHLIVWVPNNAIPYISVGDNVNV 921
               +IRA    K+  L V     V      L +I  E     +   V N  I +I+VG N  +
   Sbjct:   329 VIRAPVSVKVQQLKVHTEGGVVTTAETLMVIVPEDDTLEVTALVQNKDIGFINVGQNAII 388

   Query:   922 RYEAFPPGKFGQFTAKIMLIS 984
               + EAFP  ++G    K+  I+
   Sbjct:   389 KVEAFPYTRYGYLVGKVKNIN 409

Score = 69 (31.6 bits), Expect = 3.3e-09, Sum P(4) = 3.3e-09
Identities = 11/45 (24%), Positives = 25/45 (55%), Frame = +1

   Query:  1063 YKIVLNPEQQTIEYGEKKMPLENGMKAQSTLFLEKRKIYQWMLSP 1197
               + ++++ E+  +  G K +PL +GM   + +    R +  ++LSP
   Sbjct:   422 FNVIISIEENCLSTGNKNIPLSSGMAVTAEIKTGMRSVISYLLSP 466

Score = 48 (22.0 bits), Expect = 0.28, Sum P(2) = 0.25
Identities = 11/37 (29%), Positives = 20/37 (54%), Frame = +1

   Query:   721 TDAGRDIIIRALSDGKIDSLSVTPGQMVSVGDSLLQI 831
               T +GR   I+ + +  +  + V  G+ V  GD LL++
   Sbjct:    91 THSGRSKEIKPIENSIVKEIIVKEGESVRKGDVLLKL 127

Score = 47 (21.5 bits), Expect = 3.3e-09, Sum P(4) = 3.3e-09
Identities = 23/127 (18%), Positives = 48/127 (37%), Frame = +1

   Query:    43 PWLVISVSVFFFISFIVFVMEGTYTRRINVEGEITTWPRPVNVYSGVQGVVIKQFVTEGQ 222
               P LV    + F +   +  + G          G++T   R   +        +V +  V EG+
   Sbjct:    57 PRLVAYFIMGFLVIAFILSVLGQVEIVATANGKLTHSGRSKEIKPIENSIVKEIIVKEGE 116

   Query:   223 RIKKGDPIYLIDVSKSTSSGVVGDNKRRDIEKQLSRIGDIISRLEENKKTTLQTLEKQRI 402
                ++KGD +  +     + +  +   +        + +R   +   +E NK   L+  ++
   Sbjct:   117 SVRKGDVLLKLTALGAEADTLKTQSSLLQARLEQTRYQILSRSIELNKLPELKLPDEPYF 176

   Query:   403 QYYSAFE 423
               Q  S  E
   Sbjct:   177 QNVSEEE 183
```

```
Score = 39 (17.9 bits), Expect = 2.6e-07, Sum P(3) = 2.6e-07
Identities = 9/32 (28%), Positives = 16/32 (50%), Frame = +1

   Query:   613 QVTSLESQMQTLAAEFDNRIYQVELQRYELQK 708
               +  +L++Q   L A  +   YQ+  +  EL K
   Sbjct:   133 EADTLKTQSSLLQARLEQTRYQILSRSIELNK 164

Score = 39 (17.9 bits), Expect = 2.6e-07, Sum P(3) = 2.6e-07
Identities = 9/38 (23%), Positives = 21/38 (55%), Frame = +1

   Query:   607 LLQVTSLESQMQTLAAEFDNRIYQVELQRYELQKELVD 720
               LL++T+L ++  TL  +      ++E  RY++    ++
   Sbjct:   124 LLKLTALGAEADTLKTQSSLLQARLEQTRYQILSRSIE 161

Score = 36 (16.6 bits), Expect = 8.4e-05, Sum P(4) = 8.4e-05
Identities = 7/14 (50%), Positives = 11/14 (78%), Frame = +3

   Query:  1152 FISGKEENIPVDAI 1193
               ++ GK +NI +DAI
   Sbjct:   400 YLVGKVKNINLDAI 413

Score = 36 (16.5 bits), Expect = 3.3e-09, Sum P(4) = 3.3e-09
Identities = 9/38 (23%), Positives = 22/38 (57%), Frame = +1

   Query:   586 STQNEQNLLQVTSLESQMQTLAAEFDNRIYQVELQRYE 699
               S  ++Q + +   LE + + + A  + R+Y+ +L++ E
   Sbjct:   242 SLLHKQAIAKHAVLEQENKYVEAVNELRVYKSQLEQIE 279
```

6. MtfB

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Probability P(N)	N
gi|1197460	(U47048) MtfB [Escherichia coli]	+1	3587	0.0	1
sp|P22520|CVAB_ECOLI	COLICIN V SECRETION ATP-BINDING P...	+1	2693	0.0	1
sp|P18770|CYAB_BORPE	CYCLOLYSIN SECRETION ATP-BINDING ...	+1	383	2.7e-83	5
sp|P23702|LKTB_ACTAC	LEUKOTOXIN SECRETION ATP-BINDING ...	+1	365	5.4e-82	5
gnl|PID|e150185	(X86087) EHEC-hlyB gene product [...	+1	358	1.2e-81	4
sp|P16532|LKTB_PASHA	LEUKOTOXIN SECRETION ATP-BINDING ...	+1	377	2.4e-81	3
pir||C30169	hypothetical 79.6K protein (lktA ...	+1	377	2.4e-81	3
sp|P26760|HLYB_ACTPL	HAEMOLYSIN SECRETION ATP-BINDING ...	+1	365	3.3e-81	3
gi|457619	(L12148) pllktB [Pasteurella haem...	+1	378	1.3e-80	3
pir||S51785	RTX toxin apxIIIB - Actinobacillu...	+1	378	3.7e-79	3
sp|Q04473|RTXB_ACTPL	RTX TOXIN-III OPERON PROTEIN RTXB.	+1	378	3.7e-79	3
gi|150514	(M24197) leukotoxin (LktB) [Paste...	+1	375	7.8e-79	4
gi|505571	(X68595) secretion protein B [Act...	+1	348	9.6e-79	3
pir||A40366	hemolysin secretion protein appB ...	+1	355	2.1e-78	4
pir||S51777	xIIIB protein - Actinobacillus pl...	+1	367	1.0e-77	3
sp|P10089|HLY2_ECOLI	HAEMOLYSIN SECRETION ATP-BINDING ...	+1	367	1.9e-77	3
pir||A42255	hemolysin secretion protein hlyB ...	+1	367	2.7e-77	3
sp|P08716|HLYB_ECOLI	HAEMOLYSIN SECRETION ATP-BINDING ...	+1	367	2.7e-77	3
sp|P11599|HLYB_PROVU	HAEMOLYSIN SECRETION ATP-BINDING ...	+1	362	1.4e-76	3
pir||LEECB	hemolysin secretion protein hlyB ...	+1	367	7.4e-76	3
gi|599856	(Z46867) ABC exporter [Lactobacil...	+1	278	2.0e-74	5
gi|695620	(Z48542) SppT [Lactobacillus sake]	+1	297	6.1e-74	4
gi|972714	(L47121) ATP-dependent translocat...	+1	251	3.4e-67	5
sp|Q03727|COMA_STRPN	TRANSPORT ATP-BINDING PROTEIN COM...	+1	234	1.1e-66	4
pir||A39203	comA protein - Streptococcus pneu...	+1	233	1.5e-66	4
sp|Q00564|LCNC_LACLA	LACTOCOCCIN A TRANSPORT ATP-BINDI...	+1	250	1.1e-64	4
pir||A41464	hemolysin secretion protein CylB ...	+1	191	4.0e-63	5
gi|703126	(L40491) leucocin A translocator ...	+1	259	8.4e-61	4
pir||S52205	mesI protein - Leuconostoc mesent...	+1	162	2.7e-58	5
sp|P36497|PEDD_PEDAC	PEDIOCIN PA-1 TRANSPORT ATP-BINDI...	+1	216	5.6e-52	5
gb|I01363	Sequence 4 from Patent US 4837306...	+1	366	4.6e-50	2
sp|P21449|MDR2_CRIGR	MULTIDRUG RESISTANCE PROTEIN 2 (P...	+1	352	4.2e-47	5
sp|P21439|MDR3_HUMAN	MULTIDRUG RESISTANCE PROTEIN 3 (P...	+1	364	7.6e-47	3
sp|P06795|MDR1_MOUSE	MULTIDRUG RESISTANCE PROTEIN 1 (P...	+1	346	1.2e-46	4
sp|P45861|YWJA_BACSU	HYPOTHETICAL ABC TRANSPORTER IN A...	+1	330	1.4e-46	4
sp|P43245|MDR1_RAT	MULTIDRUG RESISTANCE PROTEIN 1 (P...	+1	356	4.3e-46	4
gi|425476	(L26287) SMDR2 [Schistosoma mansoni]	+1	385	6.8e-46	3
pir||C38696	multidrug resistance protein (clo...	+1	351	1.4e-45	3
sp|Q00449|MDR4_DROME	MULTIDRUG RESISTANCE PROTEIN HOMO...	+1	348	1.4e-45	3
pir||DVHY1C	multidrug resistance protein 1 - ...	+1	351	1.9e-45	3
sp|P21448|MDR1_CRIGR	MULTIDRUG RESISTANCE PROTEIN 1 (P...	+1	351	1.9e-45	3
gi|1065701	(Z67882) F22E10.4 [Caenorhabditis...	+1	381	3.7e-45	2
sp|P21447|MDR3_MOUSE	MULTIDRUG RESISTANCE PROTEIN 3 (P...	+1	348	4.9e-45	3
sp|Q08201|MDR2_RAT	MULTIDRUG RESISTANCE PROTEIN 2 (P...	+1	343	4.9e-45	3
sp|P23174|MDR3_CRIGR	MULTIDRUG RESISTANCE PROTEIN 3 (P...	+1	349	5.0e-45	3
pir||DVMS1A	multidrug resistance protein 1a -...	+1	345	8.3e-45	3
sp|P21440|MDR2_MOUSE	MULTIDRUG RESISTANCE PROTEIN 2 (P...	+1	350	1.3e-44	3
pir||A35671	multidrug resistance protein 1a -...	+1	345	1.3e-44	3
sp|P18768|CHVA_AGRTU	BETA-(1-->2)GLUCAN EXPORT ATP-BIN...	+1	327	2.4e-44	4
gi|159363	(L01572) P-glycoprotein [Leishman...	+1	350	3.9e-44	3
sp|P08183|MDR1_HUMAN	MULTIDRUG RESISTANCE PROTEIN 1 (P...	+1	348	4.7e-44	3
pir||A34914	multidrug resistance protein 1 - ...	+1	348	4.7e-44	3
pir||S30327	multidrug resistance protein 1 - ...	+1	243	1.5e-43	4
sp|P44407|MSBA_HAEIN	PROBABLE TRANSPORT ATP-BINDING PR...	+1	201	4.5e-43	4
gi|1139567	(D49826) Serratia marcescens LipB...	+1	199	8.7e-43	4
gi|1065700	(Z67882) F22E10.3 [Caenorhabditis...	+1	363	1.3e-42	2
sp|P27299|MSBA_ECOLI	PROBABLE TRANSPORT ATP-BINDING PR...	+1	198	2.3e-42	5
pir||A42150	P-glycoprotein atpgp1 - Arabidops...	+1	361	2.6e-42	4
pir||S25577	mt2 protein - rat	+1	344	2.6e-42	3
pir||S38400	TAP2 protein - rat >gi|407477 (X7...	+1	344	2.6e-42	3
sp|P36372|TAP2_RAT	ANTIGEN PEPTIDE TRANSPORTER 2 (AP...	+1	344	2.6e-42	3
gi|393116	(L23922) P-glycoprotein 5 [Entamo...	+1	229	2.7e-42	4
sp|P23596|PRTD_ERWCH	PROTEASES SECRETION ATP-BINDING P...	+1	182	3.1e-42	6
sp|P22638|HETA_ANASP	HETEROCYST DIFFERENTIATION ATP-BI...	+1	360	3.4e-42	2
gi|387688	(M90969) ATP-binding protein [Lac...	+1	250	6.3e-42	3
gi|290801	(L17003) member of super-family o...	+1	166	9.9e-42	5
sp|Q06034|MDR1_LEIEN	MULTIDRUG RESISTANCE PROTEIN 1 (P...	+1	341	1.1e-41	3
gi|762882	(L25849) glycoprotein P [Rattus n...	+1	198	1.2e-41	3
pir||S25576	mt2 protein - rat >pir||S38401 TA...	+1	348	1.3e-41	3
pir||S21740	probable transport protein mt2 - ...	+1	348	1.3e-41	3
gi|833699	(U17608) multidrug resistance pro...	+1	341	1.8e-41	4
gi|1065698	(Z67882) F22E10.1 [Caenorhabditis...	+1	358	2.3e-41	3
pir||S21957	P-glycoprotein pgp1 - Arabidopsis...	+1	361	2.6e-41	3
gi|405076	(U01056) P-glycoprotein 6 [Entamo...	+1	211	3.3e-41	4

```
gi|1065699          (Z67882) F22E10.2 [Caenorhabditis... +1   355  7.6e-41   3
gb|I01362|          Sequence 3 from Patent US 4837306... +1   192  8.7e-41   3
gi|159372           (L06062) multi drug resistance P-... +1   350  2.0e-40   2
gi|1236092          (Z70265) C05A9.1 [Caenorhabditis ... +1   343  2.7e-40   3
gi|576813           (M25792) P-glycoprotein [Cricetul... +1   193  4.0e-40   3
sp|P18767|NDVA_RHIME BETA-(1-->2)GLUCAN EXPORT ATP-BIN... +1   302  1.2e-39   4
sp|P37608|LCN3_LACLA LACTICIN 481/LACTOCOCCIN TRANSPOR... +1   116  1.5e-39   7
gi|1001774          (D64004) hypothetical protein [Sy... +1   300  1.9e-39   3
sp|P36619|PMD1_SCHPO LEPTOMYCIN B RESISTANCE PROTEIN P... +1   226  5.1e-39   4
gi|1143534          (Z50189) ExsA [Rhizobium meliloti]   +1   338  5.7e-39   2
pir||S30328         multidrug resistance protein 2 - ... +1   240  7.0e-39   3
gi|1236101          (Z70268) T21E8.1 [Caenorhabditis ... +1   327  3.0e-38   3
gi|1016270          (U35123) p-glycoprotein [Urechis ... +1   207  3.6e-38   3
gi|576812           (M25792) P-glycoprotein [Cricetul... +1   193  5.8e-38   4
gi|790373           (Z49126) DH11.3 [Caenorhabditis e... +1   335  6.7e-38   3
gi|1051314          (Z66562) F42E11.1 [Caenorhabditis... +1   335  8.2e-38   3
sp|P34712|MDR1_CAEEL MULTIDRUG RESISTANCE PROTEIN 1 (P... +1   333  1.5e-37   4
sp|P33311|MDL2_YEAST MDL2 PROTEIN. >pir||S42682 MDL2 p... +1   197  2.1e-37   4
gi|1235965          (Z70268) T21E8.2 [Caenorhabditis ... +1   327  4.3e-37   3
gi|1051318          (Z66562) ZK455.7 [Caenorhabditis ... +1   327  5.5e-37   3
sp|P34713|MDR3_CAEEL MULTIDRUG RESISTANCE PROTEIN 3 (P... +1   327  1.4e-36   3
sp|P36371|TAP2_MOUSE ANTIGEN PEPTIDE TRANSPORTER 2 (AP... +1   339  2.8e-36   2
gi|860725           (L42939) major histocompatibility... +1   334  2.8e-36   2
sp|Q02592|HMT1_SCHPO HEAVY METAL TOLERANCE PROTEIN PRE... +1   327  3.7e-36   3
pir||S53971         Atm1p protein (YM9952.03c) - yeas... +1   309  2.5e-35   3
sp|P40416|ATM1_YEAST MITOCHONDRIAL TRANSPORTER ATM1 PR... +1   309  2.5e-35   3
```

```
>gi|1197460 (U47048) MtfB [Escherichia coli] Length = 707
Plus Strand HSPs: Score = 3587 (1650.0 bits), Expect = 0.0, P = 0.0
                  Identities = 707/707 (100%), Positives = 707/707 (100%), Frame = +1

Query:     1 MNNNATSPLNTLLNKLEIGLRRRIPVVHQTESSECGLACLSMICGHYGRHIDLSTLRRQF 180
             MNNNATSPLNTLLNKLEIGLRRRIPVVHQTESSECGLACLSMICGHYGRHIDLSTLRRQF
Sbjct:     1 MNNNATSPLNTLLNKLEIGLRRRIPVVHQTESSECGLACLSMICGHYGRHIDLSTLRRQF 60

Query:   181 NLSALGTTLAGITEIGSQLGMETRAFSLDLNELSVLKLPCILHWEFSHFVVLVSVRKNHF 360
             NLSALGTTLAGITEIGSQLGMETRAFSLDLNELSVLKLPCILHWEFSHFVVLVSVRKNHF
Sbjct:    61 NLSALGTTLAGITEIGSQLGMETRAFSLDLNELSVLKLPCILHWEFSHFVVLVSVRKNHF 120

Query:   361 VLHDPARGRRTVGLAEMSQCFTGVALEVWPGTEFVQETMKNRVVLRTLFRSIYGLRSTLT 540
             VLHDPARGRRTVGLAEMSQCFTGVALEVWPGTEFVQETMKNRVVLRTLFRSIYGLRSTLT
Sbjct:   121 VLHDPARGRRTVGLAEMSQCFTGVALEVWPGTEFVQETMKNRVVLRTLFRSIYGLRSTLT 180

Query:   541 KIFCFSLVIEAVGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLRTAVSMIRSWS 720
             KIFCFSLVIEAVGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLRTAVSMIRSWS
Sbjct:   181 KIFCFSLVIEAVGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLRTAVSMIRSWS 240

Query:   721 SLVMETLINVQWQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTFTTSVVGAIMD 900
             SLVMETLINVQWQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTFTTSVVGAIMD
Sbjct:   241 SLVMETLINVQWQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTFTTSVVGAIMD 300

Query:   901 SIMVSGVLAMLVLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIREARASSYFME 1080
             SIMVSGVLAMLVLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIREARASSYFME
Sbjct:   301 SIMVSGVLAMLVLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIREARASSYFME 360

Query:  1081 TLYGIATIKMQGMGERRGRHWLNLKIDAINTGIRLARMDMLFSGINTFVAACDQVVILWL 1260
             TLYGIATIKMQGMGERRGRHWLNLKIDAINTGIRLARMDMLFSGINTFVAACDQVVILWL
Sbjct:   361 TLYGIATIKMQGMGERRGRHWLNLKIDAINTGIRLARMDMLFSGINTFVAACDQVVILWL 420

Query:  1261 GTSLVIDNQMTIGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIADIAMNEREAR 1440
             GTSLVIDNQMTIGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIADIAMNEREAR
Sbjct:   421 GTSLVIDNQMTIGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIADIAMNEREAR 480

Query:  1441 KPDTAMKADMYPVALETQDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMK 1620
             KPDTAMKADMYPVALETQDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMK
Sbjct:   481 KPDTAMKADMYPVALETQDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMK 540

Query:  1621 VLCGLLVPESGRVMIDGTDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEA 1800
             VLCGLLVPESGRVMIDGTDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEA
Sbjct:   541 VLCGLLVPESGRVMIDGTDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEA 600

Query:  1801 WMVECARASFIHDVIIKMPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEAT 1980
             WMVECARASFIHDVIIKMPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEAT
Sbjct:   601 WMVECARASFIHDVIIKMPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEAT 660

Query:  1981 SALDTESEYYVNQAIKQLNITRIIIAHRETTVKSADRIILLEAPARI 2121
             SALDTESEYYVNQAIKQLNITRIIIAHRETTVKSADRIILLEAPARI
Sbjct:   661 SALDTESEYYVNQAIKQLNITRIIIAHRETTVKSADRIILLEAPARI 707
```

```
>sp|P22520|CVAB_ECOLI COLICIN V SECRETION ATP-BINDING PROTEIN CVAB.
>pir||IKEC5B colicin V secretion protein cvaB - Escherichia coli
plasmid ColV >gi|41176 (X57524) cvaB protein [Escherichia coli] Length = 698
Plus Strand HSPs: Score = 2693 (1238.8 bits), Expect = 0.0, P = 0.0
                  Identities = 507/688 (73%), Positives = 601/688 (87%), Frame = +1

   Query:    34 LLNKLEIGLRRRIPVVHQTESSECGLACLSMICGHYGRHIDLSTLRRQFNLSALGTTLAG 213
               ++N L++  +RR+PV+HQTE++ECGLACL+MICGH+G++IDL  LRR+FNLSA G TLAG
   Sbjct:     9 IINLLDLRWQRRVPVIHQTETAECGLACLAMICGHFGKNIDLIYLRRKFNLSARGATLAG 68

   Query:   214 ITEIGSQLGMETRAFSLDLNELSVLKLPCILHWEFSHFVVLVSVRKNHFVLHDPARGRRT 393
               I  I  QLGM TRA SL+L+EL VLK PCILHW+FSHFVVLVSV++N +VLHDPARG R
   Sbjct:    69 INGIAEQLGMATRALSLELDELRVLKTPCILHWDFSHFVVLVSVKRNRYVLHDPARGIRY 128

   Query:   394 VGLAEMSQCFTGVALEVWPGTEFVQETMKNRVVLRTLFRSIYGLRSTLTKIFCFSLVIEA 573
               +   EMS+ FTGVALEVWPG+EF  ET++ R+ LR+L  SIYG++ TL KIFC S+VIEA
   Sbjct:   129 ISREEMSRYFTGVALEVWPGSEFQSETLQTRISLRSLINSIYGIKRTLAKIFCLSVVIEA 188

   Query:   574 VGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLRTAVSMIRSWSSLVMETLINVQ 753
               + L++PVGTQLVMDHAIPAGDRGLL+LI    LMFFILL+ A S +R+WSSLVM TLINVQ
   Sbjct:   189 INLLMPVGTQLVMDHAIPAGDRGLLTLISAALMFFILLKAATSTLRAWSSLVMSTLINVQ 248

   Query:   754 WQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTFTTSVVGAIMDSIMVSGVLAML 933
               WQSGL  HLL+LPLA+FERRK+GDIQSRF SLDTLR TFTTSV+G IMDSIMV GV  M+
   Sbjct:   249 WQSGLFDHLLRLPLAFFERRKLGDIQSRFDSLDTLRATFTTSVIGFIMDSIMVVGVCVMM 308

   Query:   934 VLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIREARASSYFMETLYGIATIKMQ 1113
               +LYGG+LT IVL FT IY+ IRL+TY  YRQ+SEE L+REARA+SYFMETLYGIAT+K+Q
   Sbjct:   309 LLYGGYLTWIVLCFTTIYIFIRLVTYGNYRQISEECLVREARAASYFMETLYGIATVKIQ 368

   Query:  1114 GMGERRGRHWLNLKIDAINTGIRLARMDMLFSGINTFVAACDQVVILWLGTSLVIDNQMT 1293
               GM   RG HWLN+KIDAIN+GI+L RMD+LF GINTFV ACDQ+VILWLG  LVIDNQMT
   Sbjct:   369 GMVGIRGAHWLNMKIDAINSGIKLTRMDLLFGGINTFVTACDQIVILWLGAGLVIDNQMT 428

   Query:  1294 IGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIADIAMNEREARKPDTAMKADMY 1473
               IGMFVAF  FRGQFS+RV SLT+FLLQLR+MSLHNERIADIA++E+E +KP+  + ADM
   Sbjct:   429 IGMFVAFSSFRGQFSERVASLTSFLLQLRIMSLHNERIADIALHEKEEKKPEIEIVADMG 488

   Query:  1474 PVALETQDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMKVLCGLLVPESG 1653
               P++LET  LS+RYDSQSAP+FS L++S+ PGESVAITGASG+GKTTLMKVLCGL  P+SG
   Sbjct:   489 PISLETNGLSYRYDSQSAPIFSALSLSVAPGESVAITGASGAGKTTLMKVLCGLFEPDSG 548

   Query:  1654 RVMIDGTDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEAWMVECARASFI 1833
               RV+I+G DIR +G+NNYH++I+CVMQDDRLFSGSIRENICGF E +DE WMVECARAS I
   Sbjct:   549 RVLINGIDIRQIGINNYHRMIACVMQDDRLFSGSIRENICGFAEEMDEEWMVECARASHI 608

   Query:  1834 HDVIIKMPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEATSALDTESEYYV 2013
               HDVI+ MPMGY+TLIGELGEGLSGGQKQRIFIARALYR+PGILFMDEATSALD+ESE++V
   Sbjct:   609 HDVIMNMPMGYETLIGELGEGLSGGQKQRIFIARALYRKPGILFMDEATSALDSESEHFV 668

   Query:  2014 NQAIKQLNITRIIIAHRETTVKSADRII 2097
               N AIK +NITR+IIAHRETT+++ DR+I
   Sbjct:   669 NVAIKNMNITRVIIAHRETTLRTVDRVI 696

>sp|P18770|CYAB_BORPE CYCLOLYSIN SECRETION ATP-BINDING PROTEIN CYAB.
>pir||BVBRCB cyaB protein - Bordetella pertussis >gi|39733 (X14199) cyaB gene product
[Bordetella pertussis] Length = 712
Plus Strand HSPs: Score = 383 (176.2 bits), Expect = 2.7e-83, Sum P(5) = 2.7e-83
                  Identities = 80/192 (41%), Positives = 121/192 (63%), Frame = +1

   Query:  1480 ALETQDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMKVLCGLLVPESGRV 1659
               ++E   +SFRY   +A    N+++ I PGE V + G SGSGK+TL +++  + V + GRV
   Sbjct:   470 SIELDRVSFRYRPDAADALRNVSLRIAPGEVVGVVGRSGSGKSTLTRLIQRMFVADRGRV 529

   Query:  1660 MIDGTDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEAWMVECARASFIHD 1839
               +IDG DI  +   +  + +  V+Q+  LF+ S+R+NI           +V  AR +  H+
   Sbjct:   530 LIDGHDIGIVDSASLRRQLGVVLQESTLFNRSVRDNIALTRPGASMHEVVAAARLAGAHE 589

   Query:  1840 VIIKMPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEATSALDTESEYYVNQ 2019
                I ++P GYDT++GE G GLSGGQ+QRI IARAL  RP +L +DEATSALD ESE+ + +
   Sbjct:   590 FICQLPEGYDTMLGENGVGLSGGQRQRIGIARALIHRPRVLILDEATSALDYESEHIIQR 649

   Query:  2020 AIKQLNITRIII 2055
                ++ +   R +I
   Sbjct:   650 NMRDICDGRTVI 661
```

```
Score = 248 (114.1 bits), Expect = 2.7e-83, Sum P(5) = 2.7e-83
Identities = 62/226 (27%), Positives = 115/226 (50%), Frame = +1

   Query:   526 RSTLTKIFCFSLVIEAVGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLRTAVSM 705
                R  + ++   SLV++ + L+ P+  Q+VMD  +        L++I VG +  IL    ++
   Sbjct:   155 RHLIGEVLLISLVLQFISLLTPLFFQVVMDKVLVNNAMETLNVITVGFLAAILFEALLTG 214

   Query:   706 IRSWSSLVMETLINVQWQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTFTTSVV 885
                IR++      + ++V+  + L+ HLL+LPLAYF+ R++GD  +R   L+ +R   T + V
   Sbjct:   215 IRTYLFAHTSSKLDVELGARLYAHLLRLPLAYFQARRVGDSVARVRELEHIRAFLTGNAV 274

   Query:   886 GAIMDSIMVSGVLAMLVLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIREARAS 1065
                  ++D +     +A++  Y   LT +VL     Y L+ L+     R+       R A
   Sbjct:   275 TVLLDVVFSVVFIAVMFFYSVKLTLVVLAALPCYFLLSLVLTPVLRRRLHVKFNRGAENQ 334

   Query:  1066 SYFMETLYGIATIKMQGMGERRGRHWLNLKIDAINTGIRLARMDML 1203
                ++ +ET+ GI T+K   +  +  R+W       +  G+ +A + ML
   Sbjct:   335 AFLVETVSGIDTVKSLAVEPQWQRNWDRQLAGYVAAGLSVANVAML 380

    Score = 64 (29.4 bits), Expect = 2.7e-83, Sum P(5) = 2.7e-83
    Identities = 13/22 (59%), Positives = 18/22 (81%), Frame = +1

   Query:  2041 TRIIIAHRETTVKSADRIILLE 2106
                T IIIAHR + V+ ADRI+++E
   Sbjct:   659 TVIIIAHRLSAVRCADRIVVME 680

Score = 58 (26.7 bits), Expect = 2.7e-83, Sum P(5) = 2.7e-83
Identities = 18/81 (22%), Positives = 31/81 (38%), Frame = +1

   Query:  1285 QMTIGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIADIAMNEREARKPDTAMKA 1464
                +MT+G  VAF +  G  +  V  L       +   +  +R+ DI     E       + A
   Sbjct:   407 RMTVGELVAFNMLSGHVTQPVIRLAQLWNDFQQTGVSMQRLGDILNCRTEVAGDKAQLPA 466

   Query:  1465 DMYPVALETQDLSFRYDSQSA 1527
                     + L+     +R D+  A
   Sbjct:   467 LRGSIELDRVSFRYRPDAADA 487

Score = 55 (25.3 bits), Expect = 2.7e-83, Sum P(5) = 2.7e-83
Identities = 12/36 (33%), Positives = 17/36 (47%), Frame = +1

   Query:    73 PVVHQTESSECGLACLSMICGHYGRHIDLSTLRRQF 180
                PV       + GL CL M+  ++G   D   LR +F
   Sbjct:     4 PVAQCASVPDSGLLCLVMLARYHGLAADPEQLRHEF 39

>sp|P23702|LKTB_ACTAC LEUKOTOXIN SECRETION ATP-BINDING PROTEIN LKTB.
>pir||S12601 lktB protein - Actinobacillus actinomycetemcomitans
>pir||A61378 leukotoxin expression protein B - Actinobacillus actinomycetemcomitans
>gi|38647 (X53955) LKTB [Haemophilus actinomycetemcomitans] Length = 707
Plus Strand HSPs: Score = 365 (167.9 bits), Expect = 5.4e-82, Sum P(5) = 5.4e-82
                  Identities = 77/188 (40%), Positives = 114/188 (60%), Frame = +1

   Query:  1492 QDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMKVLCGLLVPESGRVMIDG 1671
                +++ FRY   S  + +N+N+ I  GE + I G SGSGK+TL K++     +PE G+V+IDG
   Sbjct:   471 RNIKFRYKPDSPMILNNINLDISQGEVIGIVGRSGSGKSTLTKLIQRFYIPEQGQVLIDG 530

   Query:  1672 TDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEAWMVECARASFIHDVIIK 1851
                 D+     N   + +  V+QD+ L + SIRENI      +    ++  A+ +  HD I +
   Sbjct:   531 HDLALADPNWLRRQVGVVLQDNVLLNRSIRENIALTNPGMPMEKVIAAAKLAGAHDFISE 590

   Query:  1852 MPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEATSALDTESEYYVNQAIKQ 2031
                +  GY+T++GE G GLSGGQ+QRI IARAL   P IL  DEATSALD ESE  +   + +
   Sbjct:   591 LREGYNTVVGEQGAGLSGGQRQRIAIARALVNNPRILIFDEATSALDYESENIIMHNMHK 650

   Query:  2032 LNITRIII 2055
                +   R ++
   Sbjct:   651 ICQNRTVL 658

Score = 216 (99.4 bits), Expect = 5.4e-82, Sum P(5) = 5.4e-82
Identities = 55/206 (26%), Positives = 98/206 (47%), Frame = +1

   Query:   526 RSTLTKIFCFSLVIEAVGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLRTAVSM 705
                R    +    S+ ++   L+ P+  Q+VMD  +        L++I V L   +L    +
   Sbjct:   151 RKIFIETLIVSIFLQIFALITPLFFQVVMDKVLVHRGFSTLNVITVALAIVVLFEIILGG 210

   Query:   706 IRSWSSLVMETLINVQWQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTFTTSVV 885
                +R++       + I+V+  + L RHLL LP++YFE R++GD  +R   LD +R   T   +
   Sbjct:   211 LRTYVFAHSTSRIDVELGARLFRHLLALPISYFEARRVGDTVARVRELDQIRNFLTGQAL 270
```

```
   Query:   886 GAIMDSIMVSGVLAMLVLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIREARAS 1065
                +I+D +       A++  Y   LT +VLG    YV+  +       R+  ++   R A
   Sbjct:   271 TSILDLLFSFIFFAVMWYYSPKLTLVVLGSLPCYVIWSVFISPILRRRLDDKFARNADNQ 330

   Query:  1066 SYFMETLYGIATIKMQGMGERRGRHW 1143
                S+ +E++  I TIK   +  +    W
   Sbjct:   331 SFLVESVTAINTIKAMAISPQMTNIW 356

Score = 94 (43.2 bits), Expect = 5.4e-82, Sum P(5) = 5.4e-82
Identities = 25/104 (24%), Positives = 43/104 (41%), Frame = +1

   Query:  1243 VVILWLGTSLVIDNQMTIGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIADIAM 1422
                V+ LWLG  LVI   ++IG  +AF +  GQ    V  L       + + +   R+ D+
   Sbjct:   390 VINLWLGAHLVISGDLSIGQLIAFNMLAGQIISPVIRLAQIWQDFQQVGISVTRLGDVLN 449

   Query:  1423 NEREARKPDTAMKADMYPVALETQDLSFRYDSQSAPVFSNLNIS 1554
                +  E      ++      ++       ++ DS       NL+IS
   Sbjct:   450 SPTENNTASVSLPEIQGEISFRNIKFRYKPDSPMILNNINLDIS 493

Score = 73 (33.6 bits), Expect = 5.4e-82, Sum P(5) = 5.4e-82
Identities = 15/24 (62%), Positives = 21/24 (87%), Frame = +1

   Query:  2035 NITRIIIAHRETTVKSADRIILLE 2106
                N T +IIAHR +TVK+ADRII+++
   Sbjct:   654 NRTVLIIAHRLSTVKNADRIIVMD 677

    Score = 51 (23.5 bits), Expect = 5.4e-82, Sum P(5) = 5.4e-82
    Identities = 13/85 (15%), Positives = 36/85 (42%), Frame = +1

   Query:    91 ESSECGLACLSMICGHYGRHIDLSTLRRQFNLSALGTTLAGITEIGSQLGMETRAFSLDL 270
                +++   L  L ++   ++   I+   ++ +F++   G           LG++ R  +  +
   Sbjct:     5 KNTNLALQALEVLAQYHNISINPEEIKHKFDIDGHGLNQTKWLLAAKSLGLKVRTANKTV 64

   Query:   271 NELSVLKLPCILHWEFSHFVVLVSV 345
                + L  L LP +   +     +L+ +
   Sbjct:    65 DRLPFLHLPALAWRDDGEHFILLKI 89

>gnl|PID|e150185 (X86087) EHEC-hlyB gene product [Escherichia coli] Length = 706
Plus Strand HSPs: Score = 358 (164.7 bits), Expect = 1.2e-81, Sum P(4) = 1.2e-81
                  Identities = 77/191 (40%), Positives = 117/191 (61%), Frame = +1

   Query:  1483 LETQDLSFRYDSQSAPVFSNLNISIKPGESVAITGASGSGKTTLMKVLCGLLVPESGRVM 1662
                +E +++ FRY S     + +N+N+ I  G+ + I G SGSGK+TL K+L     +PE+G+++
   Sbjct:   468 IEFKNVRFRYSSDGNVILNNINLYISKGDVIGIVGRSGSGKSTLTKLLQRFYIPETGQIL 527

   Query:  1663 IDGTDIRSLGVNNYHKIISCVMQDDRLFSGSIRENICGFTENIDEAWMVECARASFIHDV 1842
                IDG D+          + I  V+Q++ L + SI +NI   +  +      +E AR +  HD
   Sbjct:   528 IDGHDLSLADPEWLRRQIGVVLQENILLNRSIIDNITLASPAVSMEQAIEAARLAGAHDF 587

   Query:  1843 IIKMPMGYDTLIGELGEGLSGGQKQRIFIARALYRRPGILFMDEATSALDTESEYYVNQA 2022
                I ++  GY+T++GE G GLSGGQ+QRI IARAL   P IL  DEATSALD ESE  + +
   Sbjct:   588 IRELKEGYNTIVGEQGVGLSGGQRQRIAIARALVTNPRILIFDEATSALDYESENIIMKN 647

   Query:  2023 IKQLNITRIII 2055
                + ++   R +I
   Sbjct:   648 MSRICKNRTVI 658

Score = 279 (128.3 bits), Expect = 1.2e-81, Sum P(4) = 1.2e-81
Identities = 73/302 (24%), Positives = 138/302 (45%), Frame = +1

   Query:   511 SIYGLRSTLTKIFCFSLVIEAVGLVIPVGTQLVMDHAIPAGDRGLLSLICVGLMFFILLR 690
                S+   R  L ++   S  I+ + L+ P+  Q+VMD  +         L++I +  +  IL
   Sbjct:   146 SVVKYRRILLEVLTVSAFIQFLALITPLFFQVVMDKVLVHRGFSTLNIITIAFIIVILFE 205

   Query:   691 TAVSMIRSWSSLVMETLINVQWQSGLHRHLLQLPLAYFERRKMGDIQSRFSSLDTLRTTF 870
                  ++  R++       + I+V+  + L RHLL LP++YFE R++G+  +R   LD +R
   Sbjct:   206 VILTGARTYIFSHTTSRIDVELGAKLFRHLLALPVSYFENRRVGETVARVRELDQIRNFL 265

   Query:   871 TTSVVGAIMDSIMVSGVLAMLVLYGGWLTTIVLGFTIIYVLIRLLTYNYYRQLSEESLIR 1050
                T   + +++D          ++  Y   LT ++L     YV+  L       R+  ++   +R
   Sbjct:   266 TGQALTSVLDLFFSVIFFCVMWYYSPQLTLVILLSLPCYVIWSLFISPLLRRRLDDKFLR 325

   Query:  1051 EARASSYFMETLYGIATIKMQGMGERRGRHWLNLKIDAINTGIRLARMDMLFSGINTFVA 1230
                 A   ++ +ET+  I TIK   +  +    W       + +  R+  + M        +
   Sbjct:   326 NAENQAFLVETVTAINTIKSMAVSPQMIATWDKQLAGYVASSFRVNLVAMTGQQGIQLIQ 385

   Query:  1231 ACDQVVILWLGTSLVIDNQMTIGMFVAFGVFRGQFSDRVGSLTNFLLQLRMMSLHNERIA 1410
                   V+ LW+G  LVI  +++IG  +AF +  GQ    V  L +     + +  + ER+
   Sbjct:   386 KSVMVISLWMGAHLVISGEISIGQLIAFNMLAGQVIAPVIRLAHLWQDFQQVGISVERLG 445
```

```
   Query:  1411 DI 1416
                D+
   Sbjct:   446 DV 447

Score = 70 (32.2 bits), Expect = 1.2e-81, Sum P(4) = 1.2e-81
Identities = 15/24 (62%), Positives = 21/24 (87%), Frame = +1

   Query:  2035 NITRIIIAHRETTVKSADRIILLE 2106
                N T IIIAHR +TVK+A+RII+++
   Sbjct:   654 NRTVIIIAHRLSTVKNANRIIVMD 677

Score = 60 (27.6 bits), Expect = 1.2e-81, Sum P(4) = 1.2e-81
Identities = 15/87 (17%), Positives = 37/87 (42%), Frame = +1

   Query:    94 SSECGLACLSMICGHYGRHIDLSTLRRQFNLSALGTTLAGITEIGSQLGMETRAFSLDLN 273
                SS   L  L ++  ++   ++  T+R Q+N       +          +G++ +      +
   Sbjct:     6 SSHNSLYALILLAQYHNITVNAETIRHQYNTHTQDFGVTEWLLAAKSIGLKAKYVEKHFS 65

   Query:   274 ELSVLKLPCILHWEFSHFVVLVSVRKN 354
                LS++ LP ++  +     +L  + K+
   Sbjct:    66 RLSIISLPALIWRDDGKHYILSRITKD 92

Score = 37 (17.0 bits), Expect = 2.8e-43, Sum P(3) = 2.8e-43
Identities = 7/19 (36%), Positives = 11/19 (57%), Frame = +1

   Query:   256 FSLDLNELSVLKLPCILHW 312
                +S  L  + +L LPC + W
   Sbjct:   289 YSPQLTLVILLSLPCYVIW 307
```