3 Molecular Evolution of Bacteriocins in Gram-Negative Bacteria

Milind A. Chavan and Margaret A. Riley

Summary

The study of molecular evolution has become a valuable tool in understanding the origin of life and the speciation of organisms, with the focus on changes in DNA and protein sequence and their functions. Interest in studying the molecular evolution of bacteriocins, the narrow-spectrum peptide antimicrobials, was elicited due to the broad diversity and abundance of these proteins. The availability of a large amount of data on colicins, the bacteriocins produced by the Gram-negative bacterium, *Escherichia coli*, made it a model bacteriocin to study molecular evolution. Colicins have characteristic features which make them amenable resources in the investigation of the mechanisms employed in evolution. In this chapter, we have reviewed these features of colicins and we describe models proposed to explain how these antimicrobial proteins have evolved. Further, we have described how our current understanding of colicin evolution is important to the understanding of colicin-like bacteriocins produced by other Gram-negative bacteria.

3.1 Introduction

Microbes produce a remarkable array of toxins which help them to compete in their local environments for the limited niche space and nutritional resources available. The killing breadth of these toxins ranges from quite narrow (e.g., bacteriocins are potent antimicrobials which tend to target only members of the producing species) to quite broad (e.g., classical antibiotics often target highly divergent species of bacteria). Bacteriocins, a member of the narrow-spectrum toxins, have been described as the "microbial weapon of choice", based upon their abundance and diversity among producing bacteria (Reeves 1965; Riley and Wertz 2002b). By contrast, very few microbial

Department of Biology, University of Massachusetts Amherst, Amherst, MA 01003, USA, e-mail: riley@bio.umass.edu; mchavan@bio.umass.edu

Bacteriocins: Ecology and Evolution
(ed. by M.A. Riley and M.A Chavan)

lineages produce broad-range toxins such as classical antibiotics (Berdy 1974; Hopwood and Chater 1980).

The first bacteriocin was reported in 1925 by Gratia, who observed inhibition of *Escherichia coli* ϕ by *E. coli* V. Since it killed *E. coli*, the inhibitory agent was initially referred to as "colicine" (renamed to colicin). Gratia and Fredericq generated a vast body of literature providing the very first, detailed information about the diverse family of colicin toxins (Fredericq 1957, 1963; Reeves 1965). It is largely the result of this impetus that the colicins became a model system for many subsequent studies of bacteriocin biochemistry, genetics, ecology and evolution (Riley and Gordon 1992; Riley 1993b; Braun et al. 1994; Gordon and Riley 1999; Smarda and Obdrzalek 2001; Riley and Wertz 2002a; Kirkup and Riley 2004). These studies have provided insights into the origins and function of colicins, their closest relatives (colicin-like bacteriocins produced by members of the Enterobacteriaceae), and certain types of pyocins, which are bacteriocins produced by *Pseudomonas* spp. (Parret and De Mot 2000; Riley et al. 2001; Michel-Briand and Baysse 2002). The primary focus of this chapter is to describe current knowledge regarding the evolution and diversification of colicins and their close relatives produced by Gram-negative bacteria.

3.2 Bacteriocins of Gram-Negative Bacteria

Gram-negative bacteria produce a wide variety of bacteriocins, which are specifically named after the genus (e.g., klebicins of *Klebsiella pneumoniae*) or species (e.g., colicins of *E. coli*, marcescins of *Serratia marcescens*, alveicins of *Hafnia alvei* and cloacins of *Enterobacter cloacae*) of the producing bacteria. The bacteriocins produced by *Pseudomonads* are generally referred to as pyocins.

This diversity of Gram-negative bacteriocins can be divided into three groups based on size: (1) large colicin-like (25–80 kDa) bacteriocins, (2) the much smaller microcins (<10 kDa) and (3) phage tail-like bacteriocins, which are multimeric peptide assemblies. Colicin-like large bacteriocins are SOS-inducible high molecular weight proteins (Braun et al. 1994). These bacteriocins will be described in following sections. Microcins are non-SOS-inducible low molecular weight peptides similar to the bacteriocins of Gram-positive bacteria (Baquero et al. 1978; Moreno et al. 2002). Phage tail-like bacteriocins are nuclease- and protease-resistant rod-like particles resembling a bacteriophage tail, which kill sensitive cells by depolarization of the cell membrane (Stachura et al. 1969; Kuroda and Kagiyama 1983; Nakayama et al. 2000; Strauch et al. 2003). These are proposed to be defective phages or to have originated from phages which evolved to function as bacteriocins. For example, pyocin R2 (produced by *Pseudomonas* spp.) appears to be a remnant of phage P2 whereas pyocin F2 is similar to phage lambda (Nakayama et al. 2000).

3.3 Colicins and Colicin-like Bacteriocins

Colicins are a diverse group of SOS-inducible high molecular weight bacteriocins produced by *Escherichia coli*. Colicin-encoding genes are found on plasmids, and numerous types of colicin molecules have been reported in the literature, as described in Chapter 2 (this volume). They are detected at high frequencies in natural populations of *E. coli* (Table 2.1 in Chap. 2), a feature indicative of their importance in microbial ecology (Riley and Gordon 1992).

Other members of the Enterobacteriaceae family also exhibit a high frequency (30–50%) of bacteriocin production (Pugsley 1984). Many of these bacteriocins are similar to colicins in structure and function, and share many molecular, evolutionary and ecological features as well. They are often referred to as colicin-like bacteriocins (CLBs). Historically, the interest in bacteriocins produced by, for example, *Klebsiella* sp., *Enterobacter* sp. and *Serratia* sp. has been limited to those which assist in the typing of clinical isolates. Some CLBs have been cloned and sequenced (Guasch et al. 1995; Riley et al. 2001; Wertz and Riley 2004; Chavan et al. 2005) but additional studies are required to begin to understand the diversity of CLBs. Similarly to colicins, CLBs have narrow killing spectra which are generally restricted to closely related species (Riley et al. 2003). Thus, klebicins, alveicins and marcescins have a killing range generally limited to *Klebsiella*, *Hafnia* and *Serratia* species respectively (Riley et al. 2003).

Bacteriocins produced by *Pseudomonas* spp. (pyocins) have also been extensively studied (Kuroda and Kagiyama 1983; Sano et al. 1993b; Duport et al. 1995). The pyocin genes are chromosomally encoded and are ubiquitous in *Pseudomonas*. Three types of pyocins have been described: F type, R type and S type. R- and F-type pyocins are produced by more than 90% and S-type by 70% of surveyed *P. aeruginosa* strains (Michel-Briand and Baysse 2002). Due to such high frequencies of these three types of pyocins, a *Pseudomonas* strain often produces more than one pyocin. The F and R types of pyocins are phage tail-like bacteriocins resistant to nucleases and proteases. The S-type pyocins are protease-sensitive bacteriocins similar to colicins and hence, comparison of S-type pyocins with colicins will be emphasized here.

3.3.1 Colicin Gene Organization

Colicin gene clusters consist of three tightly linked genes encoding the toxin, immunity and lysis proteins, and are usually found on plasmids. The toxin gene encodes the activity which kills the target cells. The immunity gene encodes a protein which protects the host cell from the killing action of its own colicin protein and from colicin produced by its clones (Fredericq 1957). Immunity protein binds adjacent to the active site of the colicin protein, and inhibits its activity by steric hindrance and electrostatic repulsion mechanisms. The lysis protein (also called the bacteriocin release protein) lyses the

host cell to release the expressed bacteriocin proteins outside the cell. The lysis gene is sometimes absent, particularly when more than one colicin gene cluster coexist in the same cell. This interesting aspect of co-occurring colicins is discussed in Chapter 2 (this volume).

Colicins kill target cells by pore formation, nuclease activity or by disrupting the cell wall. Figure 3.1 presents the arrangement of genes encoding pore-former and nuclease colicins. The orientation of the immunity gene with reference to the colicin genes is different for nuclease and pore-forming colicin gene clusters. In the case of pore formers (e.g., colicins A, B, K, N, E1), the immunity gene is orientated opposite to the toxin gene. The immunity and toxin genes are co-linear in nuclease colicins (e.g., colicins E2-E9), thus forming an operon consisting of toxin, immunity and lysis genes. Some nuclease colicins also have additional genes providing immunity to additional colicins. For example, the colicin E3 gene cluster contains an additional gene encoding immunity to colicin E8 (Toba et al. 1988).

The gene clusters of pore-forming and nuclease colicin-like bacteriocins show similar organization as is observed for pore-forming and nuclease colicins respectively (Riley et al. 2001; Wertz and Riley 2004; Chavan et al. 2005). Immunity genes of S-type nuclease pyocins are expressed from the same strand as in nuclease colicin gene clusters whereas the immunity gene for pyocin S5, a pore former, is expressed from the opposite strand, similar to that of pore-forming colicins. A lysis gene has not been identified for pyocin gene clusters. However, Nakayama et al. (2000) suggest that the lytic systems described in *P. aeruginosa* PAO1 for R2 and F2 pyocins may be shared by S-type pyocins. In the case of 28B, a bacteriocin produced by *Serratia marcescens*, *regA* and *regB* genes encoding phage holin and phage lysozyme-like proteins are proposed to serve in the release of bacteriocins (Ferrer et al. 1996).

Colicin expression is regulated by the SOS induction system, which is mediated by the LexA repressor binding to an inverted repeat sequence

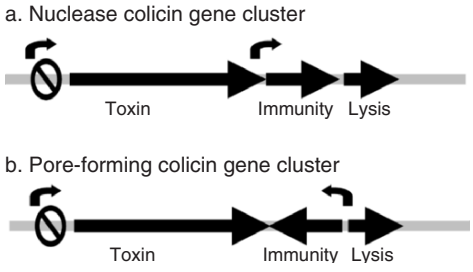


Fig. 3.1 Genetic organization of **a** nuclease and **b** pore-forming colicins. The orientation of genes encoding for killing activity, immunity and lysis proteins is depicted with *arrows*. The transcriptional starts are indicated by *curved arrows*. The LexA-binding region is indicated by ⃠

between the promoter and the ribosome binding site (Varley and Boulnois 1984). Some bacteriocins (28B and pyocins) do not have a LexA-binding box and are indirectly regulated by the SOS response via some other repressor protein (Matsui et al. 1993; Ferrer et al. 1996). The colicin and lysis genes share the same promoter whereas the immunity gene is induced from a constitutive promoter which maintains a certain basal level of immunity protein at all times to protect the producing cell.

When the SOS response is triggered in cells at times of stress, colicin genes are rapidly induced to express high levels of protein. In the case of nuclease colicins, the co-linear arrangement of the immunity and colicin genes within the gene cluster results in increased co-expression of the immunity protein which will bind to newly synthesized colicins and protect the cells from its nuclease activity. In the case of pore-forming colicins, induction does not result in increased levels of immunity protein, as the immunity gene is transcribed from the other strand. Pore-forming colicins, unlike the nuclease colicins, can kill the cells only from the exterior by punching holes in the cell membrane. Therefore, it may not be necessary for the cells to increase the levels of immunity protein during a phase of rapid colicin expression.

The colicin gene cluster consisting of toxin, immunity and lysis genes appears to have evolved to efficiently utilize the cellular resources for maximal colicin activity during colicin induction. The organization of toxin and immunity genes for pore forming ensures that resources are not wasted to express immunity protein after induction of pore-former colicin. However, cells expressing nuclease colicins are immediately protected by simultaneous induction of immunity proteins from the lethal enzymatic activity of their own colicins. This increased protection provides the cells sufficient time to produce enough colicins before their lysis.

3.3.2 Functional Domains in Colicin and CLB Proteins

The colicin and many CLB toxin proteins are organized into three functional domains: the N-terminal translocation, the central receptor binding, and the C-terminal killing domains (Fig. 3.2a). Pyocin proteins also contain these three domains but these are organized differently, and include an additional domain of unknown function (Fig. 3.2b). The receptor-binding and translocation domains of a colicin are required for uptake of colicin into sensitive cells. Together, these domains enable the colicin to recognize and enter target cells and thus, determine the specificity of colicin killing. The killing domain is responsible for the colicin activity which kills the sensitive cells by various mechanisms described below.

The interaction of a colicin molecule with the target cell is initiated by the binding of the receptor-binding domain to a specific cell surface receptor located on the outer cell surface. For example, colicins A and E1-E9 bind to the outer membrane protein BtuB, which is also known as the vitamin B12

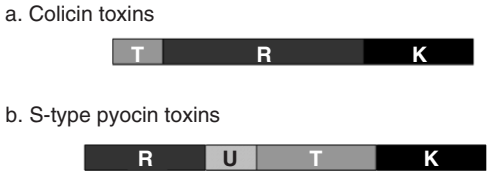


Fig. 3.2 Bacteriocin protein domain organization for **a** colicins and **b** S-type pyocins: translocation domain (*T*), receptor-binding domain (*R*), killing domain (*K*) and pyocin-specific domain (*U*), of as yet unknown function

receptor. The colicin protein is subsequently imported into the cell via the translocation domain utilizing either the TolA or TonB translocation system to move across the cell's outer membrane to reach the inner membrane (in the case of pore formers) or the cytoplasm (in the case of the nucleases). The killing domain then mediates the killing of a target cell by pore formation or nuclease activity. Pore formers (colicins A, B, K, N, S4, etc.) kill cells by creating pores in the cell membrane (Ohno-Iwashita and Imahori 1982). Nuclease colicins have DNase or RNase activities which degrade 16S rRNA or tRNAs. DNase colicins (colicins E2, E7, E8, E9) act non-specifically to digest target DNA (Toba et al. 1988; Chak et al. 1991). RNase colicins (colicins D, E3, E4, E5, E6) inactivate the protein biosynthetic machinery by targeting either 16S rRNA or tRNAs. Colicins E3, E4 and E6 have ribosomal endonuclease activity which hydrolyzes 16S rRNase (Bowman et al. 1971). Colicins D and E5 have hydrolytic activities on specific tRNA. Colicin D targets the four arginine isoacceptors (Tomita et al. 2000; Masaki and Ogawa 2002) whereas colicin E5 hydrolyses tRNAs for tyrosine, histidine, asparagine and aspartic acid, which contain a modified base, quenuine, at the wobble position of each anticodon (Ogawa et al. 1999). Additionally, a muraminidase function has been described for colicin M and pesticin activity. Muraminidase colicins degrade murein in the bacterial cell wall and thereby affect the cell's structural integrity, resulting in cell lysis (Schaller et al. 1982; Vollmer et al. 1997).

The S-type pyocins have a modular structure consisting of four domains. The S pyocin domains are, from N-terminal to C-terminal, a receptor-binding domain, a domain of unidentified function, a translocation domain, and a cytotoxic (killing) domain (Fig. 3.2b). Pyocin S1 lacks the second domain with unidentified function. As in the case of colicins, receptor-binding and translocation domains determine the species specificity whereas killing domains determine the mode of pyocin activity. A cognate immunity protein protects the pyocin-producing cell by binding to and masking the killing domain. However, further studies are required to define the receptor-binding and translocation domains of pyocin S5, which appear to have a different domain organization from that of the other S pyocins, as well as for colicin-like bacteriocins.

Initial studies indicated that pyocins S1 and S2 also inhibit lipid synthesis in sensitive cells, *P. aeruginosa* PML1516d, in addition to DNA damage (Sano

et al. 1993b). Pyocin S3 did not exhibit such an effect on lipid metabolism in sensitive strain *P. aeruginosa* PA03092. Subsequent research with pyocin S1/S2/S3 chimeras indicated that none of the chimera pyocins caused inhibition of lipid synthesis in strain PA03092 but did so in strain PML1516d (Sano et al. 1993a). This lipid synthesis inhibition in strain PML1516d was not attributable to a particular domain. The inhibition of lipid synthesis is thus a species-specific secondary event in strain PML1516d, due to pyocin activities.

The domains of colicins and pyocins act in concert to kill sensitive cells. The receptor-binding and translocation domains exploit the host cellular infrastructure for the import of colicin into the cell. The activity of the cytotoxic domain then mediates the killing of the host/sensitive cell.

3.4 Models of Colicin Evolution

Riley and co-workers have extensively studied the evolution of colicins, and it is the result of these efforts that colicins are being recognized as a model system for the evolution of Gram-negative bacteriocins (Riley 1993a, 1993b, 1998; Riley et al. 1994, 2000; Tan and Riley 1996, 1997a; Riley and Wertz 2002a, 2002b). Two mechanisms – diversifying selection and recombination – have been proposed to explain how these molecules have evolved (Tan and Riley 1997b). According to the diversifying selection model, positive selection creates novel colicins by a series of point mutations which initially generate novel immunities and, subsequently, novel killing domains. The diversifying recombination model explains how diversity is created by the recombination of different colicin domains. Newly discovered colicin-like bacteriocins from related bacteria also exhibit characteristic features of molecular evolution proposed for colicins in prior studies (Riley et al. 2001; Wertz and Riley 2004; Chavan et al. 2005). Similarities observed in killing domains of colicins and CLBs indicate a common origin for these domains. Lateral transfer of genes or DNA segments may be responsible for the spread of killing domains.

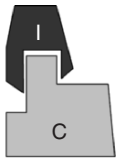
3.4.1 Diversifying Selection

The colicin E3 and E6 gene clusters exhibit an interesting pattern of divergence. These two gene clusters have 95% identity within DNA sequences spanning their genes encoding for toxins and immunity proteins. However, the changes in nucleotide bases are not evenly distributed. Most mutations have accumulated within the region spanning the colicin-killing domain and the 5′ half of the immunity gene. Thus, the immunity region and the immunity-binding region of the colicin gene exhibit high levels of polymorphism, which are not observed for the translocation and receptor-binding domains of colicins. A similar pattern was recorded in the divergence of colicins

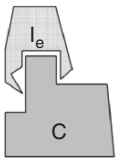
E2 and E9. The role of positive selection was invoked, and a diversifying selection model was proposed to explain these patterns of nucleotide divergence in these two pairs of nuclease colicins (Riley 1993a, 1993b).

Riley and co-workers have proposed a "diversifying selection" hypothesis in which sequence diversification is thought to occur in two stages, as shown in Fig. 3.3. First, a point mutation occurs in the immunity gene, giving it a broadened immunity function (the ability to bind multiple colicins) and thereby conferring a selective advantage ensuring higher fitness than for its wild-type neighbours in a population producing multiple colicins. Second, a compensating mutation in the toxin gene of the colicin occurs in the immunity protein-binding site, thereby generating a "super killer" phenotype (Riley 1993b). Superkillers kill ancestral colicin-producing cells, since the immunity gene of the ancestral strain is no longer capable of binding the new toxin (Fig. 3.3d). At the same time, the newly evolved colicin-producing cell is protected from the ancestral colicin, since its immunity protein can still bind to the ancestral toxin.

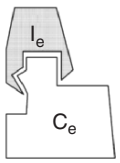
The mechanism by which elevated mutation rates are achieved in colicin gene clusters may be linked to the physiology of the cell during SOS induction. During SOS, the expression of the error-prone DNA polymerase Pol IV is elevated, leading to an 800-fold increase in the mutation rate of replicated plasmids (Kim et al. 1997). Colicin plasmids may also mutate rapidly during this phase, leading to the evolution and selection of a new colicin gene cluster, as proposed in the diversifying selection model for colicin evolution.



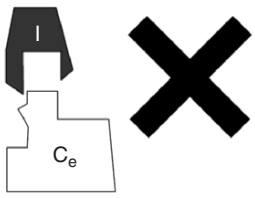
(a) Ancestral colicin (C) and immunity proteins (I)



(b) Mutation(s) in immunity gene. Evolved immunity protein (I_e) still binds ancestral colicin



(c) Mutations in colicin gene. Selection of new colicin (C_e) that specifically binds to new immunity



(d) Ancestral immunity can not bind to new colicin. Cells with ancestral colicin and immunity are no longer protected from new colicin.

Fig. 3.3 A model depicting diversifying selection for colicins

According to the diversifying selection model, a new toxin–immunity protein combination evolves by modification of the protein-binding interactions between the toxin and immunity proteins. Thus, new killing domains have evolved by generating new immunity specificity. The co-evolution of colicins and associated immunity proteins proposed in this model ensures that high-affinity binding interactions between the toxin and immunity proteins are selected so that the cells are not killed by their own toxins. This model of evolution is particularly well documented in nuclease colicins, of which the short killing domains (85–110 amino acid residues) have limited flexibility for diversification because mutations affecting the functional domain may render these colicins inactive.

3.4.2 Diversifying Recombination

All characterized, pore-former colicin proteins have high levels of localized sequence similarity to other pore-former colicins, creating a patchwork of shared and divergent sequences. The locations of the different patches frequently correspond to the different functional domains of the proteins. These observations led to the proposal that the diversity of colicins is increased by recombination events which exchange different domains (Braun et al. 1994; Tan and Riley 1997b). Such domain-based shuffling between bacteriocins is responsible for much of the variability observed among pore formers and also in nuclease colicins (e.g., colicins E6 and E7).

Examples of mix-and-match patterns of colicin domains are shown in Fig. 3.4. This is particular striking in colicins B and D, which have almost identical N-terminal translocation and receptor-binding domains. Their killing domains, however, have been selected from different sources. Colicin B has a colicin Y-like pore-forming domain whereas colicin D has a tRNase domain similar to that of klebicin D and colicin E4. Comparison of colicin 5, 10 and K sequences indicate the role of recombination events in creating new colicins by exchanging domains (Pilsl and Braun 1995). Colicin 5 has a colicin 10-like N-terminal region but a colicin K-like killing domain (Fig. 3.4). Colicins Ia and Ib also have identical N-terminal domains but their killing domains have only 51% identity.

Thus, there are two phases in colicin evolution. Initially, novel colicins are created by diversifying selection. If the novel colicins are successful, they become abundant and further diversities are subsequently created by recombination events (Riley and Wertz 2002b).

3.4.3 Evolution of Colicin-like Bacteriocins

Colicins and CLBs possess some common features: gene cluster organization, tri-domain organization of toxin, SOS-mediated expression, and shared

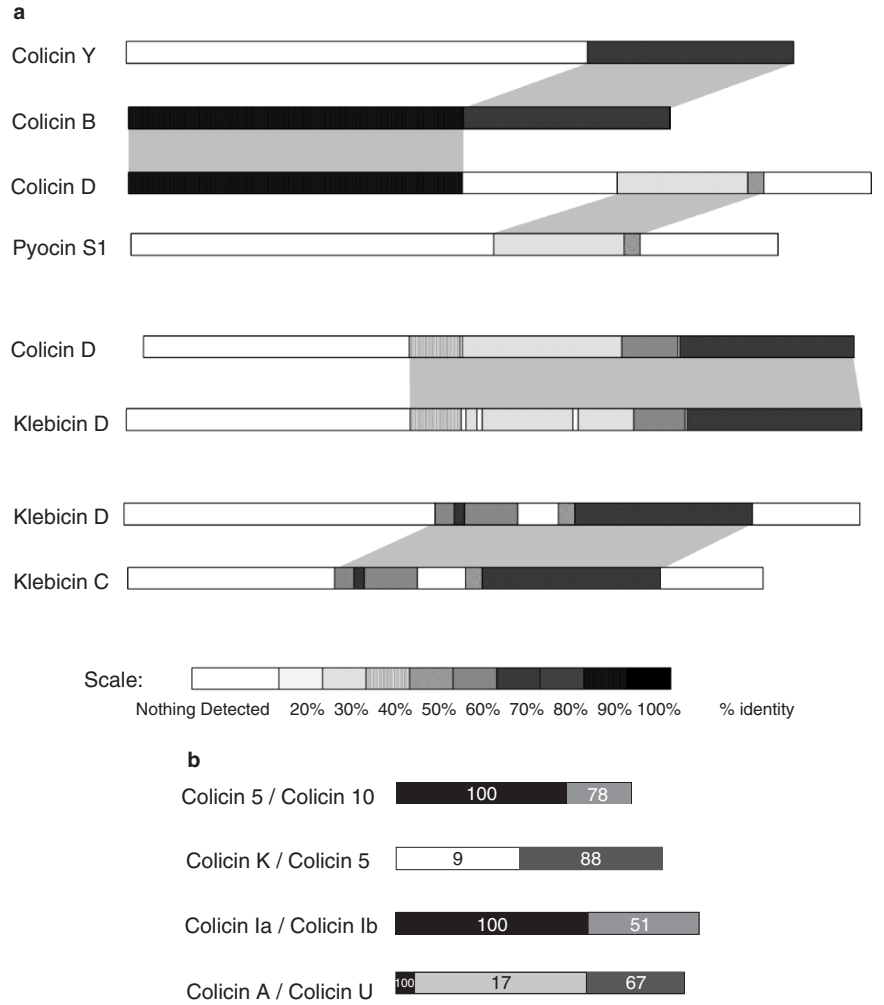


Fig. 3.4 A model depicting diversifying recombination for colicins. **a** Shared regions among colicins Y, B, D, pyocin S1, and klebicins C and D. The *scale* indicates the percentage identities between the shared regions. The *grey highlight* emphasizes the shared region between two individual activity proteins. **b** Pairs of colicins with regions of significant similarity indicated by % identity

killing domains. Riley and colleagues investigated whether or not the diversifying selection and recombination models developed to describe the molecular evolution of colicins could be extended to CLBs produced by other members of the Enterobacteriaceae family to which *E. coli* belongs. If applicable, the vast knowledge available in the colicin literature could be applied to bacteriocins produced by related species such as *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp., *Hafnia* sp. and *Citrobacter* sp. Sequence data of

bacteriocins cloned from *Klebsiella* and *Hafnia* sp. indicate that these are indeed similar to colicins in mode of action, protein structure, and genetic organization (Riley et al. 2001; Wertz and Riley 2004; Chavan et al. 2005).

The most recent illustration of diversifying recombination is seen from recently published sequences of klebicins (all of which have nuclease activity) and alveicins. The klebicin B gene cluster shares sequence similarity with colicin A in the 5′ regulatory region and 3′ lysis gene (Riley et al. 2001). Although the source of its translocation and receptor-binding domains are unknown, it has a nuclease colicin-like killing domain (most similar to pyocin S1) and immunity gene (most similar to colicin E9 immunity). Klebicin C has a klebicin D-like receptor-binding domain but a colicin E1-like translocation domain and a colicin E4-like DNase domain (Chavan et al. 2005). Conversely, klebicin D shows a colicin D-like rRNase domain. Similarly, alveicins A and B of *Hafnia alvei* have similar translocation and killing domains but their receptor-binding domains are clearly different (Wertz and Riley 2004).

The klebicin killing domains are homologues of known killing domains – klebicins B, C and D are similar to pyocin S1, colicin E4 and colicin D respectively. These examples exhibit the diversifying selection model proposed for their colicin counterparts, as their evolutionary history seems to involve mutations in their immunity genes in conjunction with corresponding changes in their killing domains, as predicted in the diversifying selection mechanism.

The influence of diversifying recombination is not limited to the closely related bacteriocins of enteric bacteria. The S pyocins of *P. aeruginosa* are speculated to have evolved as a result of recombination between several pore-former and nuclease colicins with other, as yet uncharacterized bacteriocins (Sano et al. 1993a, 1993b). Thus, altering the domain structure of the protein, as seen for pyocins which have switched the receptor recognition and translocation domains relative to the order found in colicins, has not limited the influence of diversifying recombination.

3.5 Evolution of Colicin Killing Domains

The killing domains are the function modules of colicins which catalyze deleterious changes in the sensitive cell, leading to death. Various killing mechanisms are employed by colicins, as described in the sections above. Sequence data of klebicins, S-type pyocins, alveicins, marcescin and cloacins indicate that CLBs exploit the same killing functions as those used by colicins.

Alignments of selected colicin and CLB DNase, rRNase and pore-forming domains are shown in Fig. 3.5. Based on these alignments, Tables 3.1 and 3.2 show percentage similarities and divergence between various pairs of DNase, 16S rRNase and pore-forming colicins respectively. A phylogenetic tree inferred from sequences of nuclease domains of colicins, CLBs and pyocins is

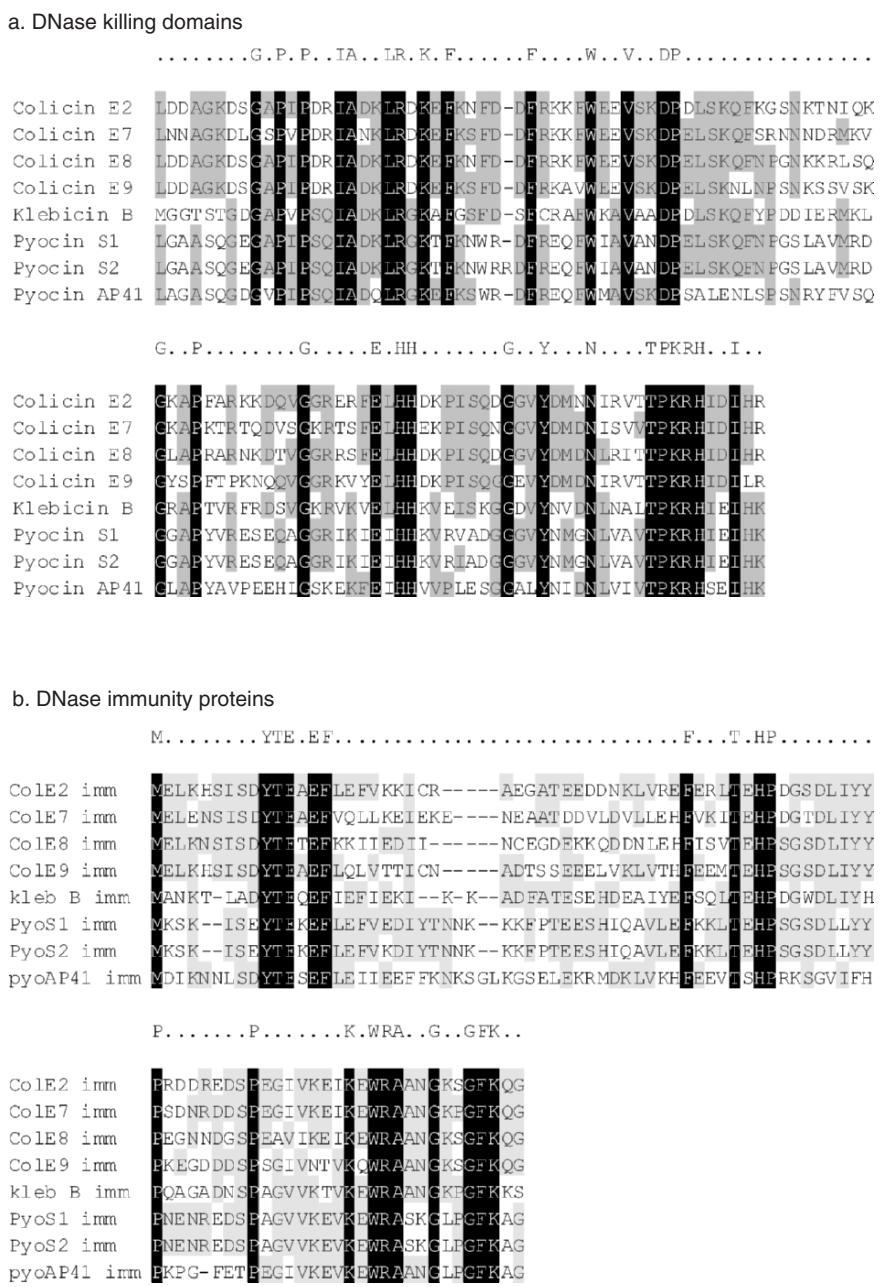


Fig. 3.5 Amino acid alignments of killing domains and immunity proteins of colicins and colicin-like bacteriocins. Killing domains of DNase (**a**), 16S rRNase (**c**), and pore-forming (**e**) domains and their respective immunity proteins (**b**, **d**, **f**) are shown. Amino acid residues conserved in the majority of sequences are highlighted in *grey* whereas those conserved in all sequences are shown in *black*

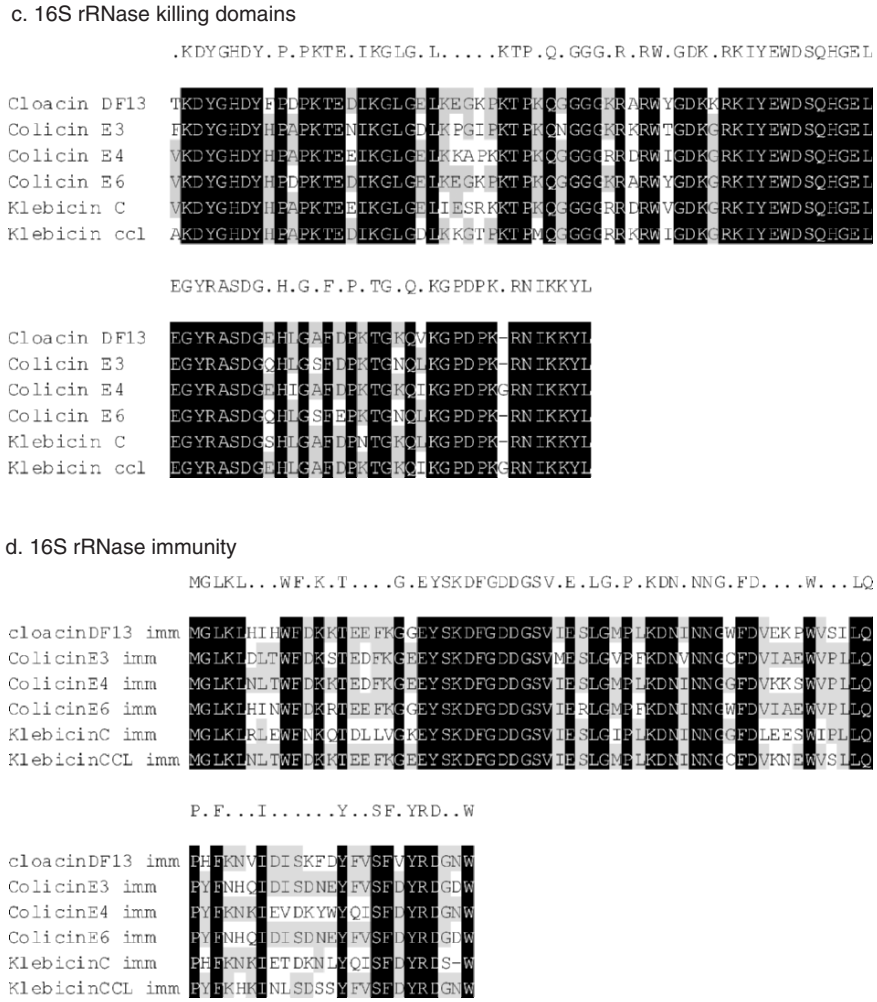
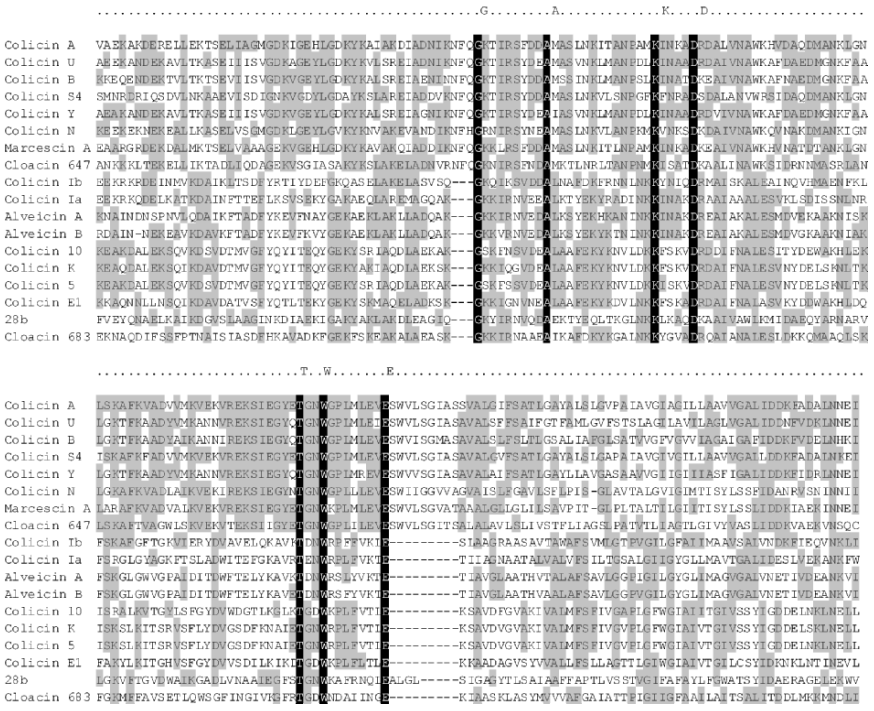


Fig. 3.5 Continued

shown in Fig. 3.6. The outcome clustered different nuclease activities – DNase domain, rRNase activity, colicin D-like tRNase activity and colicin E5-like tRNase activity – into specific groups. DNase domains of pyocins S1, S2 and AP41 are homologous to killing domains of DNase colicins. Pyocins (S1, S2 and AP41) form a separate cluster to that of colicin DNase domains (E2, E7, E8, E9), with klebicin B clustering closer to pyocins than to colicins (Riley et al. 2001). The corresponding immunity proteins of pyocins S1, S2 and AP41 and colicins E2, E7, E8 and E9 also exhibit significant similarities with each other. Thus, the DNase domain–immunity gene combination of colicins and pyocins appears to share an evolutionary pathway.

e. Pore forming domains



f. Pore forming immunity

Fig. 3.5 Continued

Table 3.1 Pairwise distance of DNase killing domains and associated immunity proteins from colicins and colicin-like bacteriocins

	Colicin E2, colicin E2 imm	Colicin E7, colicin E7 imm	Colicin E8, colicin E8 imm	Colicin E9, colicin E9 imm	Klebicin B, klebicin B imm	Pyocin S1, pyocin S1 imm	Pyocin S2, pyocin S2 imm	Pyocin AP41, pyocin AP41 imm
DNase killing domains (percent similarity)								
Colicin E2	***	71.8	82.7	77.3	47.3	50.0	50.9	43.6
Colicin E7	35.3	***	71.8	65.5	50	47.3	48.2	42.7
Colicin E8	19.7	35.3	***	74.5	50.9	53.6	54.5	48.2
Colicin E9	27.1	46.1	31.1	***	44.5	46.4	47.3	45.5
Klebicin B	87.4	79.9	77.5	95.7	***	58.2	59.1	46.4
Pyocin S1	79.9	87.4	70.6	90.1	60.4	***	99.1	57.3
Pyocin S2	77.5	84.8	68.5	87.4	58.4	0.9	***	57.3
Pyocin AP41	98.7	101.7	84.8	92.9	90.1	62.3	62.3	***
Associated immunity proteins (percent divergence)								
Colicin E2 imm	***	67.4	57.6	68.6	56.6	60.7	61.9	49.4
Colicin E7 imm	42.6	***	62.4	59.3	50	49.4	50.6	46.5
Colicin E8 imm	61.5	51.9	***	55.3	49.4	50.6	49.4	48.8
Colicin E9 imm	40.6	58	66.8	***	51.8	52.4	52.4	49.4
Klebicin B imm	63.7	79.9	81.5	75.2	***	56.0	54.8	46.4
Pyocin S1 imm	55.1	81.4	78.3	73.7	65.3	***	98.9	46.5
Pyocin S2 imm	52.8	78.3	81.5	73.7	68.0	1.2	***	45.3
Pyocin AP41 imm	81.4	89.7	83.1	81.4	89.9	89.7	93.2	***

Table 3.2 Pairwise distance of 16S rRNase killing domains and associated immunity proteins from colicins and colicin-like bacteriocins

	Cloacin DF13, cloacin DF13 imm	Colicin E3, colicin E3 imm	Colicin E4, colicin E4 imm	Colicin E6, colicin E6 imm	Klebicin C, klebicin C imm	Klebicin CCL, klebicin CCL imm
16S rRNase killing domains (percent similarity)						
Cloacin DF13	***	84.2	85.3	91.6	84.2	87.4
Colicin E3	17.8	***	83.2	89.5	83.2	87.4
Colicin E4	16.5	19.1	***	84.2	90.5	90.6
Colicin E6	9.0	11.4	17.8	***	85.3	85.3
Klebicin C	17.8	19.1	10.2	16.5	***	85.3
Klebicin CCL	13.9	13.9	10.0	16.5	16.5	***
Associated immunity proteins (percent divergence)						
Cloacin DF13 imm	***	70.6	76.5	78.8	66.7	78.8
Colicin E3 imm	37.3	***	74.1	87.1	63.1	80
Colicin E4 imm	28.3	31.8	***	71.8	77.4	83.5
Colicin E6 imm	24.9	14.2	35.4	***	63.1	78.8
Klebicin C imm	43.9	50.5	27.0	50.5	***	66.7
Klebicin CCL imm	24.9	23.3	18.6	24.9	43.9	***

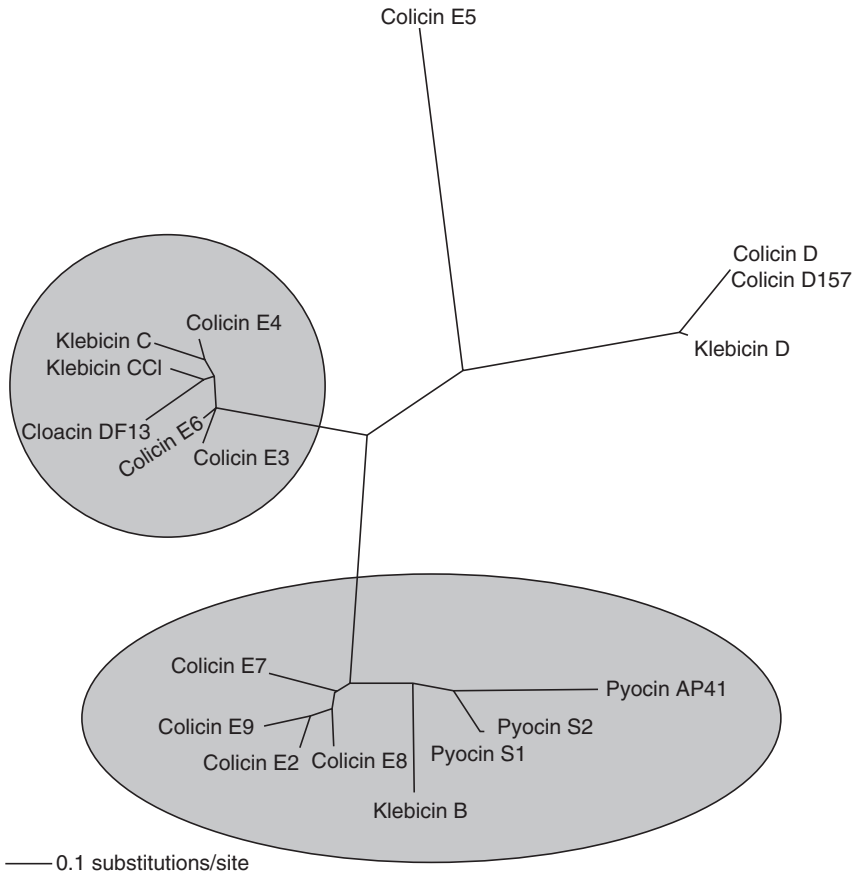


Fig. 3.6 Maximum-likelihood phylogram of nuclease killing domains of colicins and colicin-like bacteriocins. Bootstrap values >50% are indicated

The rRNase domains (colicins E3, E4 and E6, cloacin DF13, klebicin D and CCL) form a tight cluster with very short branches in the inferred phylogenetic tree (Fig. 3.6). This domain is highly conserved (Tables 3.1 and 3.2, Fig. 3.5), compared to the DNase and pore-forming domains. The tRNase domain of colicin D and klebicin D are closely related to each other whereas that of colicin E5, which has a t-RNase activity distinct from that of the former two, forms a stand-alone branch.

Pyocin S3 has DNase activity but has no sequence homology to DNase domains of colicins or other pyocins currently characterized (Duport et al. 1995). Therefore, it can be speculated that the pyocin S3 killing domain has evolved independently from that of other DNase domains. Its cognate immunity protein, which is larger than other DNase immunity proteins, appears to have co-evolved with the new DNase domain to provide protection to the pyocin S3-producing cell.

A maximum-likelihood tree inferred for the pore-forming domains is shown in Fig. 3.7. The pore-forming domains form two distinct groups: type A (e.g., colicins E1, 5, 10, K, Ia and Ib, alveicins A and B, cloacin 683) and type B (colicins A, B, N, S4, U and Y, marcescin A and cloacin 647). A bacteriocin from *Serratia*, marcescin A, is most closely related to colicins N and A in terms of level of similarity. However, colicin A clusters with colicins Y, U and S4, with which it has a higher level of similarity. Alveicins A and B produced by *Hafnia alvei* are related to colicin Ia, a type B pore former.

3.6 Evolution of the Translocation and Receptor-Binding Domains

Nature has opted for multiple mechanisms to kill target cells using pore formation, DNase, rRNase, tRNase or muramidase activity. Similarly, bacteriocins have exploited multiple species-specific receptors and import functions found in bacterial cells to mediate recognition of the target cell and access target molecules.

The translocation and receptor-binding domains of colicins do not show significant cross-species presence and function, as is observed for killing

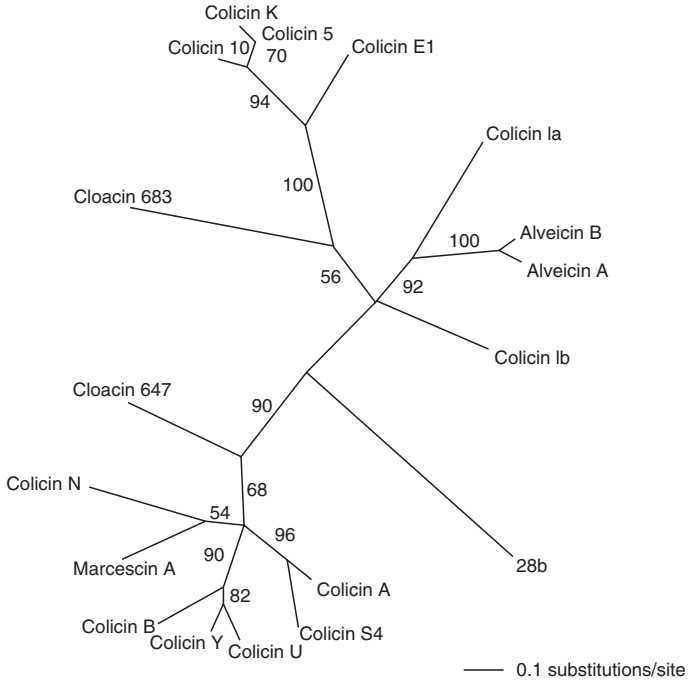


Fig. 3.7 Maximum-likelihood phylogram of pore-forming killing domains of colicins and colicin-like bacteriocins. Bootstrap values >50% are indicated

domains which are freely exchanged between colicins and CLBs. Thus, killing domains appear to be transferred more frequently than the target-defining (specifying) domains for translocation and receptor binding. The CLBs have no (or significantly lower) similarity with well-characterized translocation and receptor-binding domains of colicins, in contrast to that observed for the killing domains (Riley et al. 2001; Wertz and Riley 2004; Chavan et al. 2005). These domains have evolved in CLBs so as to be more adept in exploiting the receptors and translocation mechanisms of their own species. This has resulted in the evolution of colicins and CLBs which kill very closely related bacteria. It appears that within their ecological niches, bacteria selectively develop toxins to control closely related populations which have similar environmental requirements, rather than addressing competition from different genera. In other words, bacterial strains appear to be more threatened by siblings than by cousins.

There are some exceptions to the narrow killing ranges seen in most CLBs. Killing activity across a genera does exist, one such example being 28B produced by *Serratia marcescens* which targets *E. coli*. The sequence of the translocation domain of 28B is remarkably similar to that of colicins A and U, which also kill *E. coli*.

The translocation domains of S-type pyocins, colicins E2, E3 and cloacin DF13 are similar to each other. However, chimeric constructions of pyocin and colicin domains do not result in expected exploitation of the translocation system across the species. Thus, these domains are rather species-specific, and their similarities to each other appear to have no functional significance (Kageyama et al. 1996).

Marcescin A, also produced by *Serratia marcescens*, appears to have a novel translocation domain which has probably diversified to utilize a parallel translocation system or employ a different system in sensitive strains of *Serratia* (unpublished data). Such species specificity of translocation domains was also observed in alveicins A and B (Wertz and Riley 2004) and klebicins (Riley et al. 2001; Chavan et al. 2005). Similar species-specific diversity is found in their receptor-binding domains. Whereas the associated receptors have been characterized for all colicins, those targeted by CLBs have not been investigated to date. The receptor-binding domains of klebicins C and D have 45% protein sequence similarity to the corresponding domain of colicin D, indicating that these bacteriocins bind to similar proteins in their target species. On the other side of the spectra, the receptor-binding domains of klebicin B, alveicins A and B, and marcescin A are considered to be novel.

3.7 Evolution of Colicin Regulatory Sequences

Colicins are expressed from a SOS response regulated promoter. SOS regulation of a gene is mediated by binding of LexA repressor to a 20-bp palindrome (5′-TACTGTATATATATACAGTA-3′) sequence, referred to as

the LexA-binding box (Walker 1984). However, regulatory regions of colicins contain two overlapping LexA-binding boxes, which is considered a characteristic feature of a colicin promoter (Parker 1986; Riley et al. 2001). The only instances of non-colicin genes with colicin-like dual-overlapping LexA-binding boxes are found in three prophage genes in *S. typhi* and *S typhimurium* chromosomes. There are a few exceptions among colicins – cloacin DF13, marcescin A and klebicin D all have single LexA-binding boxes, and colicins Ia and Ib contain a second degenerate LexA box (Varley and Boulnois 1984). LexA repressor binding regions are absent in regulatory sequences of pyocins and bacteriocin 28B, even though these toxins are inducible by DNA-damaging agents. Pyocins are regulated by two genes, *prtN* and *prtR* (Matsui et al. 1993). In the case of bacteriocin 28B, a CLB produced by *Serratia*, SOS induction is mediated indirectly by a transcriptional activator protein of which the expression is repressed by SOS regulation (Ferrer et al. 1996).

A comparison of promoter sequences starting from the –35 box through to the start codon of colicins and CLBs reveals few highly conserved regions. The –35 box is most conserved and matches the consensus (5′ TTGACA 3′) whereas the –10 box shows only 50% match to the consensus (5′ TATAAT 3′; Fig. 3.8). The LexA-binding box is another conserved element which is followed by T-rich segments, and finally by the RBS (ribosome binding site). Most of the colicin promoters cluster together whereas the CLB promoters are quite divergent. The promoters of colicin A, klebicin B and a newly sequenced cloacin 683 (unpublished data) have a leader sequence between the LexA-binding boxes and the RBS. Interestingly, all these bacteriocins were sequenced from non-*E. coli* species. Klebicin B and cloacin 683 are expressed by *Klebsiella pneumoniae* and *Enterobacter cloacae* respectively, and colicin A was isolated from *Citrobacter freundii*.

Among all CLBs, the pesticin (produced by *Yersinia pestis*) promoter is most closely related to colicin promoters. The alveicin A/B promoters also share significant similarity with colicins. The bacteriocin 28B promoter is very different from the colicin promoters, though its translocation domain is similar to that seen in colicins A and U.

3.8 Colicin D: A Possible Intermediate Between Pyocins and Colicins

The modular design of colicins and pyocins provides flexibility for generating new bacteriocins by recombination mechanisms. As described above, colicin B is a classic example, with colicin D-like translocation and receptor-binding domains and a colicin A/Y-like pore-forming domain. Similarly, translocation and DNase domains of pyocins S1 and S2 are homologous whereas their receptor-binding domains have been acquired from different sources. Thus,

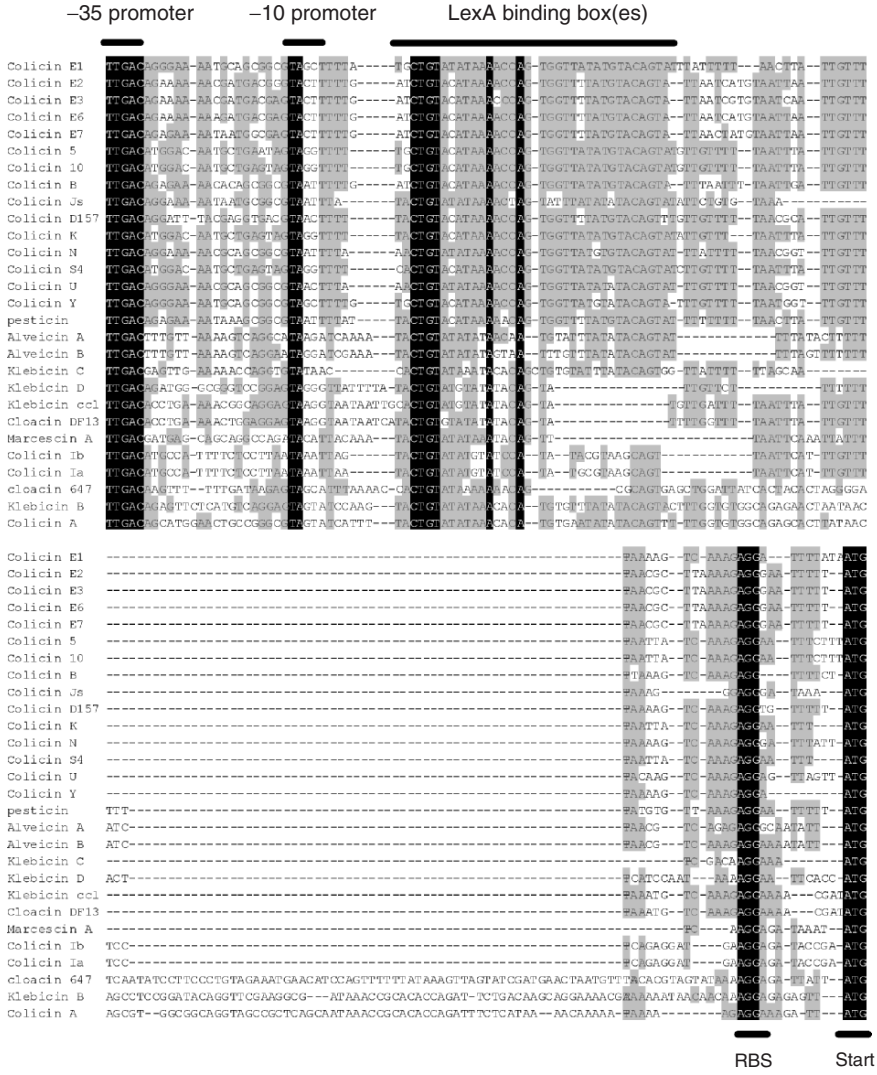


Fig. 3.8 Nucleotide alignment of regulatory regions of colicins and colicin-like bacteriocins. Nucleotides conserved in all or a majority of sequences are highlighted in *black* or *grey* respectively. The positions of –35 and –10 promoter regions, the LexA-binding regulatory sequence, the RBS (ribosomal binding Shine Dalgarno region) and start codons are indicated

nature has created a pool of candidates for each domain which can be mixed with one another. The cytotoxic domains are obviously shared between colicins and pyocins. However, sequences of colicin D and other colicin-like bacteriocins indicate that there has been more sharing between the different bacteriocins. The N-terminal 313 amino acid residues of colicin D are 95%

identical to the colicin B translocation and receptor-binding domains. The tRNase domain of colicin D consists of 97 aa residues in its C-terminal region (Takahashi et al. 2006). Thus, colicin D has a complete set of domains needed for its function. However, it contains an additional domain (~290 aa residues) between the central receptor-binding domain and the cytotoxic domain which has some similarity with the translocation domain of pyocins S1, S2, S3 and AP41. In all cases (colicin D and pyocins), this domain is located towards the N-terminus of the killing domain and hence, it is tempting to hypothesize that a translocation domain:cytotoxic domain combination was transferred from one bacteriocin to another (most probably from pyocin to colicins or CLBs). A similar organization is observed in klebicins B, C and D, all of which have a pyocin translocation-like domain adjacent to their killing domain. Klebicin B also has significant levels of similarity with the N-terminal translocation domain of colicin A whereas klebicin D has a colicin E1-like translocation domain. These klebicins also have a regulatory region similar to that of colicins. It is not clear if the original function of this second domain has been retained or lost. Another interesting feature of klebicin bacteriocins observed recently is the presence of an additional gene in the klebicin operon. This gene is located upstream of the activity gene in both the klebicin C and D operons. The encoded protein shares sequence similarity to the C-terminal domain of the phage tail fibre-like gene. Experimental evidence indicates that it is required for klebicin D activity (Chavan et al. 2005). This reinforces the idea that nature is continuing to experiment with different killing strategies.

The function of a second domain found in pyocins S2, S3 and AP41 also has not yet been determined. It could be a vestigial domain, similarly to the one described above for colicin D and klebicins. Perhaps, as a phage evolves to specialize as an R- or F-type pyocin, these new domains/genes provide additional function to increase the fitness of the host cell, thereby giving them an edge over their competitors.

3.9 Conclusions

Colicin expression results in lysis of the producer cell, due to co-expression of a lysis protein which mediates the release of colicin into the extra-cellular environment. It is obvious that such a suicidal mechanism is costly for the cell, and therefore raises questions regarding the need for bacterial cells to maintain colicin-encoding genes. One hypothesis is that colicins are being used by selfish plasmid systems for their own maintenance, as a parasite in bacterial hosts. Those cells which cure themselves of the plasmid also lose immunity and thus are killed by the colicins produced by neighbour siblings. On the other hand, colicins have also been implicated as a defence mechanism in competition for niche space and resources.

In some cases, the colicin and lysis genes form an operon tightly regulated by the SOS system, which responds to significant DNA damage. Thus, induction of colicin and co-induction of lysis genes may occur only in damaged cells, resulting in the cell's death, and thus may be considered similar to apoptosis in eukaryotic cells. Experimental evidence suggests that expression of colicin is induced in only a small fraction of the population (Mulec et al. 2003). These colicin-expressing cells eventually die but produce enough colicin to kill related, but competing, cells. Thus, a fraction of colicin-harbouring cells display altruistic behaviour by "sacrificing themselves" for the larger benefit of their clonal kin. The altruistic behaviour of colicin producers has elicited considerable interest and warrants further consideration.

The narrow killing spectra of colicins and CLBs provide additional points of consideration concerning the necessity of these bacteriocins for the cell. If the colicin-harbouring plasmid was indeed a selfish parasitic system, then these bacteriocin genes should have moved to multiple genera by a series of horizontal transfer events, as seen with antibiotic-resistant genes. For example, the plasmid encoding klebicin B could have moved from *Klebsiella pneumoniae* to *Pseudomonas* sp. or *Citrobacter* sp. However, killing activities across the genera are uncommon. Rather, we observe kin discrimination by bacteriocin-producing strains. So, why should colicin-producing *E. coli* kill its own kin, rather than individuals from a different species or genus? Maximum competition is observed between cells with more similar nutritional and niche requirements. In addition, the presence of a diverse assemblage of other bacterial species and genera is critical for the health of the environmental niche as a whole. Since colicins, and numerous other bacteriocins, have persisted for millions of years, the benefit of these actions must outweigh the consequences of cells being sacrificed in the process.

References

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

Berdy J (1974) Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Adv Appl Microbiol 18:309–406

Bowman CM, Dahlberg JE, Ikemura T, Konisky J, Nomura M (1971) Specific inactivation of 16S ribosomal RNA induced by colicin E3 in vivo. Proc Natl Acad Sci USA 68:964–968

Braun V, Pilsl H, Gross P (1994) Colicins: structures, modes of action, transfer through membranes, and evolution. Arch Microbiol 161:199–206

Chak KF, Kuo WS, Lu FM, James R (1991) Cloning and characterization of the ColE7 plasmid. J Gen Microbiol 137:91–100

Chavan M, Rafi H, Wertz J, Goldstone C, Riley MA (2005) Phage associated bacteriocins reveal a novel mechanism for bacteriocin diversification in *Klebsiella*. J Mol Evol 60:546–556

Duport C, Baysse C, Michel-Briand Y (1995) Molecular characterization of pyocin S3, a novel S-type pyocin from *Pseudomonas aeruginosa*. J Biol Chem 270:8920–8927

Ferrer S, Viejo MB, Guasch JF, Enfedaque J, Regue M (1996) Genetic evidence for an activator required for induction of colicin-like bacteriocin 28b production in *Serratia marcescens* by DNA-damaging agents. J Bacteriol 178:951–960

Fredericq P (1957) Colicins. Annu Rev Microbiol 11:7–22

Fredericq P (1963) On the nature of colicinogenic factors: a review. J Theor Biol 4:159–165

Gordon DM, Riley MA (1999) A theoretical and empirical investigation of the invasion dynamics of colicinogeny. Microbiology 145(3):655–661

Guasch JF, Enfedaque J, Ferrer S, Gargallo D, Regue M (1995) Bacteriocin 28b, a chromosomally encoded bacteriocin produced by most *Serratia marcescens* biotypes. Res Microbiol 146:477–483

Hopwood DA, Chater KF (1980) Fresh approaches to antibiotic production. Philos Trans R Soc Lond B Biol Sci 290:313–328

Kageyama M, Kobayashi M, Sano Y, Masaki H (1996) Construction and characterization of pyocin-colicin chimeric proteins. J Bacteriol 178:103–110

Kim SR, Maenhaut-Michel G, Yamada M, Yamamoto Y, Matsui K, Sofuni T, Nohmi T, Ohmori H (1997) Multiple pathways for SOS-induced mutagenesis in *Escherichia coli*: an overexpression of dinB/dinP results in strongly enhancing mutagenesis in the absence of any exogenous treatment to damage DNA. Proc Natl Acad Sci USA 94:13792–13797

Kirkup BC, Riley MA (2004) Antibiotic-mediated antagonism leads to a bacterial game of rock-paper-scissors in vivo. Nature 428:412–414

Kuroda K, Kagiyama R (1983) Biochemical relationship among three F-type pyocins, pyocin F1, F2, and F3, and phage KF1. J Biochem (Tokyo) 94:1429–1441

Masaki H, Ogawa T (2002) The modes of action of colicins E5 and D, and related cytotoxic tRNases. Biochimie 84:433–438

Matsui H, Sano Y, Ishihara H, Shinomiya T (1993) Regulation of pyocin genes in *Pseudomonas aeruginosa* by positive (prtN) and negative (prtR) regulatory genes. J Bacteriol 175:1257–1263

Michel-Briand Y, Baysse C (2002) The pyocins of *Pseudomonas aeruginosa*. Biochimie 84:499–510

Moreno F, Gonzalez-Pastor JE, Baquero MR, Bravo D (2002) The regulation of microcin B, C and J operons. Biochimie 84:521–529

Mulec J, Podlesek Z, Mrak P, Kopitar A, Ihan A, Zgur-Bertok D (2003) A cka-gfp transcriptional fusion reveals that the colicin K activity gene is induced in only 3 percent of the population. J Bacteriol 185:654–659

Nakayama K, Takashima K, Ishihara H, Shinomiya T, Kageyama M, Kanaya S, Ohnishi M, Murata T, Mori H, Hayashi T (2000) The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. Mol Microbiol 38:213–231

Ogawa T, Tomita K, Ueda T, Watanabe K, Uozumi T, Masaki H (1999) A cytotoxic ribonuclease targeting specific transfer RNA anticodons. Science 283:2097–2100

Ohno-Iwashita Y, Imahori K (1982) Assignment of the functional loci in the colicin E1 molecule by characterization of its proteolytic fragments. J Biol Chem 257:6446–6451

Parker RC (1986) Mitomycin C-induced bidirectional transcription from the colicin E1 promoter region in plasmid ColE1. Biochim Biophys Acta 868:39–44

Parret A, De Mot R (2000) Novel bacteriocins with predicted tRNase and pore-forming activities in *Pseudomonas aeruginosa* PAO1. Mol Microbiol 35:472–473

Pilsl H, Braun V (1995) Strong function-related homology between the pore-forming colicins K and 5. J Bacteriol 177:6973–6977

Pugsley AP (1984) The ins and outs of colicins. Part I. Production, and translocation across membranes. Microbiol Sci 1:168–175

Reeves P (1965) The bacteriocins. Bacteriol Rev 29:25–45

Riley MA (1993a) Molecular mechanisms of colicin evolution. Mol Biol Evol 10:1380–1395

Riley MA (1993b) Positive selection for colicin diversity in bacteria. Mol Biol Evol 10:1048–1059

Riley MA (1998) Molecular mechanisms of bacteriocin evolution. Annu Rev Genet 32:255–278

Riley MA, Gordon DM (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(7):1345–1352
Riley MA, Wertz JE (2002a) Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie 84:357–364
Riley MA, Wertz JE (2002b) Bacteriocins: evolution, ecology, and application. Annu Rev Microbiol 56:117–137
Riley MA, Tan Y, Wang J (1994) Nucleotide polymorphism in colicin E1 and Ia plasmids from natural isolates of *Escherichia coli*. Proc Natl Acad Sci USA 91:11276–11280
Riley MA, Cadavid L, Collett MS, Neely MN, Adams MD, Phillips CM, Neel JV, Friedman D (2000) The newly characterized colicin Y provides evidence of positive selection in pore-former colicin diversification. Microbiology 146(7):1671–1677
Riley MA, Pinou T, Wertz JE, Tan Y, Valletta CM (2001) Molecular characterization of the klebicin B plasmid of *Klebsiella pneumoniae*. Plasmid 45:209–221
Riley MA, Goldstone CM, Wertz JE, Gordon D (2003) A phylogenetic approach to assessing the targets of microbial warfare. J Evol Biol 16:690–697
Sano Y, Kobayashi M, Kageyama M (1993a) Functional domains of S-type pyocins deduced from chimeric molecules. J Bacteriol 175:6179–6185
Sano Y, Matsui H, Kobayashi M, Kageyama M (1993b) Molecular structures and functions of pyocins S1 and S2 in *Pseudomonas aeruginosa*. J Bacteriol 175:2907–2916
Schaller K, Holtje JV, Braun V (1982) Colicin M is an inhibitor of murein biosynthesis. J Bacteriol 152:994–1000
Smarda J, Obdrzalek V (2001) Incidence of colicinogenic strains among human *Escherichia coli*. J Basic Microbiol 41:367–374
Stachura I, McKinley FW, Leidy G, Alexander HE (1969) Incomplete bacteriophage-like particles in ultraviolet-irradiated haemophilus. J Bacteriol 98:818–820
Strauch E, Kaspar H, Schaudinn C, Damasko C, Konietzny A, Dersch P, Skurnik M, Appel B (2003) Analysis of enterocoliticin, a phage tail-like bacteriocin. Adv Exp Med Biol 529:249–251
Takahashi K, Ogawa T, Hidaka M, Ohsawa K, Masaki H, Yajima S (2006) Purification, crystallization and preliminary X-ray analysis of the catalytic domain of the *Escherichia coli* tRNase colicin D. Acta Crystallogr Sect F Struct Biol Crystallogr Commun 62:29–31
Tan Y, Riley MA (1996) Rapid invasion by colicinogenic *Escherichia coli* with novel immunity functions. Microbiology 142(8):2175–2180
Tan Y, Riley MA (1997a) Nucleotide polymorphism in colicin E2 gene clusters: evidence for nonneutral evolution. Mol Biol Evol 14:666–673
Tan Y, Riley MA (1997b) Positive selection and recombination: major molecular mechanisms in colicin diversification. Trends Ecol Evol 12:348–351
Toba M, Masaki H, Ohta T (1988) Colicin E8, a DNase which indicates an evolutionary relationship between colicins E2 and E3. J Bacteriol 170:3237–3242
Tomita K, Ogawa T, Uozumi T, Watanabe K, Masaki H (2000) A cytotoxic ribonuclease which specifically cleaves four isoaccepting arginine tRNAs at their anticodon loops. Proc Natl Acad Sci USA 97:8278–8283
Varley JM, Boulnois GJ (1984) Analysis of a cloned colicin Ib gene: complete nucleotide sequence and implications for regulation of expression. Nucl Acids Res 12:6727–6739
Vollmer W, Pilsl H, Hantke K, Holtje JV, Braun V (1997) Pesticin displays muramidase activity. J Bacteriol 179:1580–1583
Walker GC (1984) Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol Rev 48:60–93
Wertz JE, Riley MA (2004) Chimeric nature of two plasmids of *Hafnia alvei* encoding the bacteriocins alveicins A and B. J Bacteriol 186:1598–1605