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

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

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 poreformer 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. 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 \otimes

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 cyto-toxic (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 highaffinity 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 poreformer 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

a. DNase killing domains

	G.P.P.IA.LR.K.FFW.V.DP
Colicin E2 Colicin E7 Colicin E8 Colicin E9 Klebicin B Pyocin S1 Pyocin S2 Pyocin AP41	LDD AG KDS G ABT PDR IA DK LRD KE EKN FD - DE RKK FW EE VSK DE DLS KO FKG SN KT NI OK LNN AG KDL G SEV PDR IA NK LRD KE EKS FD - DE RKK FW EE VSK DE EL SK OF SR NN ND RMKV LDD AG KDS G AFT PDR IA DK LRD KE EKN FD - DE RKK FW EE VSK DE EL SK OFN PGN KKRL SO LDD AG KDS G AFT PDR IA DK LRD KE EKS FD - DE RKA VW EE VSK DE EL SKNL NP SN KS SV SK MGGT STG DG AFV PSO IA DK LRD KE EKS FD - DE RKA VW EE VSK DE EL SKNL NP SN KS SV SK LGAA SOG EG AFT PSO IA DK LRG KAFGSFD - SF CRAFW KA VAADE DLS KO FYP DD I E RMKL LGAA SOG EG AFT PSO IA DK LRG KT EKNW R- DE REO FW IA VANDE EL SK OFN PGS LA VMRD LGAA SOG EG AFT PSO IA DK LRG KT EKNW RR DE REO FW IA VANDE EL SK OFN PGS LA VMRD LGAA SOG EG AFT PSO IA DK LRG KT EKNW RR DE REO FW MA VSK DE SALENLSP SN RY FV SO
-	GPGE.HHGYNTPKRHI
Colicin E2 Colicin E7 Colicin E8	GKAP FAR KK DOVGGRER HEIHHDK PI SODGOVYDMNN IRVTTPKR HID I HR CKAP KTR TO DVSCK RTS HEIHHEK PI SONGOVYDMIN IS VVTPKR HID I HR CLAP RAR NKDTVGGRRS HEIHHDK PI SODGOVYDMIN LRITTPKR HID I HR
Colicin E9 Klebicin B Pyocin S1 Pyocin S2	CYSEFT PKNOOVCGRKVYBLHHIDKPISOCCEVYDMIN IRVTTPKRHIDILR CRAETVRFRDSVCKRVKVBLHHKVEISKGCDVYNVDNLNALTPKRHIEIHK CGAEYVRESEQACGRIKIBHHKVRVADGCGVYNMCNLVAVTPKRHIEIHK CGAEYVRESEQACGRIKIBHHKVRLADGCGVYNMCNLVAVTPKRHIEIHK
Pyocin AP41	CLAPYAVPEEHICSKEKED THHVVPLESCCALYNI INLVI VTPKRHSETHK

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b. DNase immunity proteins
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ColE2 imm	MELKHSIS	dyteace le fv kk	I CR AEGA	AT EEDDNKLVRE FE	R L <mark>T</mark> E <mark>HP</mark> DGS DL IYY
ColE7 imm	MELENSIS	EYTE AEF VQLLKE	IEKENEA#	AT DDVLDVLLEH FV.	K I <mark>T EHP</mark> DG TDL I Y Y
ColE8 imm	MELKNSISI	DYTETEFKKII ED	IINCEG	GDEKK QD DN LE H F I	S V <mark>T EHP</mark> SG SD LIYY
ColE9 imm	MELKHSIS	d yte A df LQL V TT.	I CNADTS	SEELVKLVTHE	EM <mark>TEHP</mark> SGSDLIYY
kleb B im	m MANKT-LA	d yte q<mark>df</mark>iefiek	I K- K AD FA	TESE HDEAIYE S	QL TEHP DGWDLIYH
PyoS1 imm	KSKISI	EYTEKEFLEFVED	I YTN <mark>NK KKF</mark> F	TEESHIQAVLE <mark>B</mark> K	KL <mark>TEHP</mark> SGSDLLYY
PyoS2 imm	KSKISI	E YTE KEF LE FV KD	I YT NNK – – KKF F	PTEESHIQAVLE F K	KL <mark>TEHP</mark> SGSDLLYY
pyoAP41 i	mm MDIKNNLS	DYTESEFLEIIEE	F FKNKS GLKGS E	LEKR MDK LV KH F E	EV <mark>T</mark> SHP RK SGV I FH

P......P.....K.WRA..G..GFK..

ColE2 imm	PRDDREDSPEGIVKEIKE <mark>WRA</mark> ANCKS <mark>GFK</mark> QG
ColE7 imm	ESDNRDDSEEGIVKEIKE <mark>WRA</mark> AN <mark>C</mark> KE <mark>GFK</mark> QG
ColE8 imm	BEGNNDGSEEAVIKEIKEWRAANCKS <mark>GFK</mark> QG
ColE9 imm	EKEGD DD S <mark>E</mark> SGI VNT V <mark>K</mark> QWR AAN <mark>C</mark> KS <mark>GF K</mark> QG
kleb B imm	PQAGADNSPAGVVKTVKE <mark>WRA</mark> AN <mark>C</mark> KP <mark>GFK</mark> KS
PyoS1 imm	ENENREDS FAGVVKEV <mark>K</mark> E <mark>WRA</mark> SK <mark>C</mark> LF <mark>GFK</mark> AG
PyoS2 imm	ENE NR EDS PAGV VKEV <mark>K</mark> E <mark>WR A</mark> SK <mark>C</mark> LP <mark>GF K</mark> AG
pyoAP41 imm	EKPG– FET EEGI VKEV <mark>K</mark> E <mark>WRA</mark> ANC LE <mark>GFK</mark> AG

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*

c. 16S rRNase killing domains

.KDYGHDY, P.PKTE.IKGLG.L....KTP.Q.GGG.R.RW.GDK.RKIYEWDSQHGEL

Cloacin DF13	3 TKD YG HDY FEDPKTEDIKGLGELKEGKEKTEK QG GG GKRARWYGDKKRKIY EWD SQ HGE I
Colicin E3	FKD YGHDY H PAPK TENIKGLG <mark>D IK PGI F</mark> KT F <mark>K CN</mark> GGCKRKRW TGDKG RK IY EWD SQHGE I
Colicin E4	VKD YG HDY <mark>H PA</mark> PK TEEIKGLGE IKKA PKKT EKCG GGC <mark>RR DRW I</mark> GDKG RK IY EWD SQHGE I
Colicin E6	VKD YG HDY <mark>H P</mark> DPK TE <mark>D</mark> IKGLG <mark>E IKEGKE</mark> KT E <mark>K C</mark> GGG <mark>KRARN Y</mark> GD KG RK IY EWD SQHGE I
Clebicin C	WKDYGHDY <mark>H PAPKTEE</mark> IKGLG <mark>ELIE</mark> SRKKTEKCCGGCRRDRWWGDKCRKIYEWDSQHGEI
Clebicin ccl	l a <mark>kdyghdyheapktedikglo</mark> dikkgtp <mark>ktemogggggrrkrwigdk</mark> grkiyewdsohgei
(lebicin C (lebicin ccl	VKDYGHDYH PAPKTEEIKGLGELIESR KKTFKCGGGGRRERWVGDKGRKIYEWDSQHG AKDYGHDYH PAPKTEDIKGLGDLKKGTPKTFNCGGGGRRKRWIGDKGRKIYEWDSQHG

EGYRASDG.H.G.F.P.TG.Q.KGPDPK.RNIKKYL

Cloacin	DF13	EGYRA	SDGE HL	GAFDP	K TG KQV	KG PD PK	RNIKKYL
Colicin	Ε3	EGYRA	SDGQHL	GSFDP	K TG NQL	KG PD PK	RNIKKYL
Colicin	E4	EGYRA	SDGEHI	GAFDP	K TG KQI	KG PD PK	RNIKKYL
Colicin	Ε6	EGYRA	SDGQHL	GSFEP	K TG N <mark>Q</mark> I	KG PD PK-	RNIKKYL
Klebicir	n C	EGYRA	SDG <mark>S</mark> HL	GAFDP	N TC KQI	KG PD PK-	RNIKKYL
Klebicir	n ccl	EGYRA	SDGEHL	CAFDP	K TG KQI	KG PD PK	RNIKKYL

d. 16S rRNase immunity

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	MGLKLWF.K.TG.EYSKDFGDDGSV.E.LG.P.KDN.NNG.FDWLQ
cloacinDF13 imm ColicinE3 imm ColicinE4 imm ColicinE6 imm KlebicinC imm KlebicinCCL imm	MG LKUHIHWEDKKTEE FKGGEYSKDFGDDGSVID SLGMEIKDNINN GWFDVEK PWVSILQ MG LKUDLTWEDKSIEDFKGEEYSKDFGDDGSVMESLGVERKDNINN GCFDVI AEWVFILQ MG LKUNLIWEDKKTEDFKGEEYSKDFGDDGSVIESLGWEIKDNINN GGFDVKKSWVFILQ MG LKUHINWEDKRTEE FKGGEYSKDFGDDGSVIERLGWERKDNINN GGFDUESWIFLLQ MG LKURLEWENK OIDLLVGKEYSKDFGDDGSVIESLGIEDKKDNINN GGFDLEESWIFLLQ MG LKURLEWENK OIDLLVGKEYSKDFGDDGSVIESLGIEDKKDNINN GFDLEESWIFLLQ
	P.FIYSF.YRDW
cloacinDF13 imm ColicinE3 imm ColicinE4 imm ColicinE6 imm KlebicinC imm	EH FKNVIDISKFDYFVSFVYRIGNW FYFNHÇIDISDNEYFVSFDYRIGDW FYFKNKIEVDKYWYQISFDYRIGNW FYFNHÇIDISDNEYFVSFDYRIGDW FYFNHÇITISDNEYFVSFDYRIGDW FFFKNKIETDKIYQISFDYRIS-W
KlebicinCCL imm	PYEKHKINLSDSSYFVSEDYRLGNW

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.

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e. Pore forming domains

	G		A	к	.D
Colicin A	VAEKAKDERELLEKTSELIAGMGDKIGEHLGDKYKAIAKDIADNIKNFOC	TIRSFDDA	MAS LN KIT AN PAM	INKAI	RD AL VNAWKHVD AQDMANKLGN
Colicin U	AE EKAND EKAVLTKASE II IS VGD KAGE YLGD KY KVL SR EIADNI KNFQ	TIRSYDE	MAS VN KLMAN PDL	INAAI	RDAI VNAWKAFDAEDMGNKFAA
Colicin B	KKE QE ND EK TVL TK TSE VI ISVGD KV GE YLGD KY KAL SR EI A EN I NN FQ G K	TIRSYDDA	MSS IN KLMAN PSL	INATI	KEAI VNAWKAFNAEDMGNKFAA
Colicin S4	SMNR DRIQS DVLNKAAEVISDIGN KVGDYLGDAYKSLAREIAD DVKNFQCK	TIRSYDDA	MAS LN KVL SNP GF	FNRAL	S DALAN VWRSI DAQ DMANKLGN
Colicin Y	AEA KANDEK AVLTKASE II ISVGD KVGE YLGD KY KALSRE IAGNI KNFOC	TIRSYDE	IAS VN KLMAN PDL	INAAL	RDVIVNAWKAFDAEDMGNKFAA
Colicin N	KE EK EKNEK EAL LKASELVS GMGD KLGE YLGV KY KNVAK EVAND I KNFH C R	NIRSYNE	MAS LN KVL AN PKM	VNKSI	KDA IVNAWKOVNAKDMANKIGN
Marcescin A	A EAARGRDEK DALMKTSELVAAAGEKVGEHLGDKYKAVAKQIAD DIKNFQ <mark>C</mark> K	KLRS FDD	MAS LN KIT LNP AM	INKAL	KEAI VNAWKHV NAT DTANKLGN
Cloacin 647	7 ANKKKLTEKELLIKTADLIQDAGEKVSGIA SAKYKSLAKELADNVRNFC <mark>C</mark> K	NIRSFND	MKTLNR LTAN PNM	I SATI	KAALINAWKSIDRNNMASRLAN
Colicin Ib	EE KR KRDE IN MV KD AIKLTS DF YR TI YD EFGKQAS ELA KE LA SV SQC	QIKSVDD	LNA FD KFRNN LNK	YNIO	RMAI SKALEAI NQVHMAEN FKL
Colicin Ia	EE KR KODE LK AT KD AIN FTTEF LK SVS EKY GA KAE OLAR EMA GOAKC	KIRNVEE	LKT YEK YRADI NK	INAKI	RAAIAAALESVKLSDISSNLNR
Alveicin A	KNAINDN SPNVLODA IK FTADFYK EV FNAY GE KAE KLAK LLADOAKCK	KIRNVEDA	LKS YEKHKAN INK	INAKI	REAIAKALESMOVEKAAKNISK
Alveicin B	RDAIN-NEKEAVKDAVKFTADFYKEVFKVYGE KAEKLAK LLADQAKC	KVRNVE DA	LKS YEK YK TN INK	INAKI	REAI AKALESMOVGKAAKN IAK
Colicin 10	KE AK DAL EK SÇV KD SVDTMVGF YQYI TEQYGE KYSR IAQDLA EKAKC	K FN SV DE	LAA FE KY KNV LDK	FSKV	RD DI FNA LE Ŝ IT YDEWAKH LE K
Colicin K	KE AQ DAL EK SQI KD AVDTMV GF YQ YI TEQY GE KYAKI AQDLA EK SKC	K IQG VD EA	LAA FE KY KNV LDK	FSKVI	RDAIFNALE SVNYDELSKNLTK
Colicin 5	KE AK DAL EK SÇVKD SVDTMV GE YQ YI TEQY GE KY SR IAQDLA EK AKC	K FS SV DE	LAA FE KY KNV LDK	ISKV	RDAIFNALESVNYDELSKNLTK
Colicin El	KKAQNNLLN SQI KDAVDATV SFYQTLTEKY GE KY SKMAQ ELADKSKC	KIGNVNE	LAA FE KY KDV LNK	FSKAL	RDAI FNA LASV KY DDWAKH LDQ
28b	FVEY QNAELKAI KDGVSLAAGIN KDIAEKI GAKYAKLAK DLEAGIQC	Y IRNVQ D	EKTYEQLTKGLNK	lkaqı	KAAIVAWIKMIDAEQYARNARV
Cloacin 683	3 EKNAQD IF SSFPTNAIS IASDFHKAVAD KFGE KFSKE AKALA EA SKC	KIRNAAE	IKAFDKYKGALNK	YGVAT	ROATANALESLOKKOMAACLSK
	E				

Colicin A	LSKAFKVADVVMKVEKVREKSIEGYE <mark>n</mark> gn	GPIMLEV	SWVLSGIASSVALGIFS AT LGAYALS LGVPAIAVGIACILLAAV VGALIDDK FADAINNE I
Colicin U	LG KT FKA AD YVMKANNVR EKSIEG YOT GN	GPLMLEI	SWVLSGIA SAVALSFFS AIF GTFAML GVFSTSLAGILAVILAGL VGAL ID DNFVDKINNE I
Colicin B	LGKTFKAADYAIKANNIREKSIEGYO <mark>R</mark> GN	GPLMLEV	SWVISGMA SAVALS LESLTIGSALIA EGLSA TVVG EVGVVIA GAIGA FIDDKEV DE LNHKI
Colicin S4	ISKAFKFADVVMKVEKVREKSIEGYE	GPIMLEV	SWVLSGIA SAVALGVFS AT LGAYALS IGAPA IAVGI VGILLAAV VGALLDD KFADAIN KEI
Colicin Y	LG KT FKA AD YVMKANNVREKSIEG YOTGN	GP LMR EV	SWVV SGIA SAVALAI FSAT LGAYLLA VGASA AVVGI IGI IIASF IGALI DD KFI DRINNE I
Colicin N	LGKAFKVADLAIKVEKIREKSIEGYN	GPLLLEV	SWIIGGVVAGVAISLEGAVLSELPIS-GLAVTALGVIGIMTISYLSSEIDANRVSNINNII
Marcescin A	. LARAF KVAD VALKVEKVREK SIEG YE <mark>R</mark> GN	KPLMLEV	SWVLSGVATAAALGLIGLI LSAVP IT -GLPLTALTI LGI ITI SYLSSLI DD KIAEK INNE I
Cloacin 647	LSKAFTVAGWLSKVEKVTEKSIIGYE	GPLILEV	SWVLSGIT SALALAV LSLIVSTFLIAGSL PATVTLIAGTLGIVY VAS LIDD KVAEK VNSQC
Colicin Ib	FSKAFGFTGKVIERYDVAVELQKAVK	ØRPFFVKI	SLAAGRAASAVTAWAFSVMLGT PVGILGFAI IMAAVSALVNDKFIEQVNKLI
Colicin Ia	FSRGLGYAGKFTSLADWITEFGKAVR	RPLFVKI	TILAGNAATALVALVFS ILTGSALGI IG YGLLMAVTGALI DESLVEKANKFW
Alveicin A	FSKGLGWVGPAIDITDWFTELYKAVK	RS LYV KT	TIAVGLAATHVTALAFSAVLGGPIGILGYGLIMAGVGALVNETIVDEANKVI
Alveicin B	FSKGLGWVGPAIDITDWFTELYKAVE	RSFYVKT	TIAVGLAATH VAALAF SAVLGG PV GIL GYGLI MAGVGAI VNETI VD EANK VI
Colicin 10	ISRALKVTGYLSFGYDVWDGTLKGLK	KPLFVTI	9KSAVD FGVAKI VA 1M FS FIV GA PLG FWGIAI I TGI VSS YI GD DE LNKLNE LL
Colicin K	ISKSLKITSRVSFLYDVGSDFKNAIB	RPLFVTI	KSAVDVGVAKI VALMFS FIV GV PLG FWGIAI VT GI VSS YI GD DELSKLNELL
Colicin 5	ISKSLKITSRVSFLYDVGSDFKNAIE	RPLFVTI	9KSAVDVGVAKI VALMES FIV GV PLC FWGIAI VT GI VSS YI GD DELNKLNEIL
Colicin El	FAKYLKITGHVSFGYDVVSDILKIKI	KPL FL TL	KKAADAGVS YVVALL FS LLAGTT LGIWGIAI VT GI LC SYIDKNKLNT INEVL
28b	LG KV FTG VD WAIKGADLVNAAIE G FSTGN	MKA FR NQ I	ALGLSIGAJYTLSAIAAFFAPTLVSSTVGIFAFAYLFJWATSYIDAERAGELEKWV
Cloacin 683	FGEMEFAVSETLQWSGFINGIVKGFREGD	M NDAI ING	K IAAS KLAS YMVVVAFGA IA TT PIGI IG FAAI LAI TS AL ITD DLMKKMND LI

f. Pore forming immunity

Colicin A imm	MMNEHS IDTDNRKANNALYLFIIGLIPLLCIFVVYYKTPDALLLRKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAILYLTFKIRFIGALLARKIATSTENLPSITSSYNPLMTKVMDIYCKTAPFLAFTIFT
Colicin U imm	MQKEHNDNKQLNNMLSWIAFAGTIPFFIILIICKLNPNLYLTNIIPDSIINIPSVISSYNPIMTKLMDIYGKSAPLLAIITFILQFRHAMMANAMANAMANAMANAMANAMANAMANAMANAMANAM
Colicin B imm	MTSNKDKNKKANEILYAFSIIGIIPIMAILILRINDPYSQVLYYLYNKVAFLPSITSLHDPVMTTLMSNYNKTAPVMGILVFLCTYKT
Colicin S4 imm	MMIDEHSIDIDNRKANNLLYLFMVIGVIPLLCILAVYYTNPDNLFLHTIATSTENIPSITSAYNPLMTKVMDIYCKTAPSLAILFILTFKTR
Colicin Y imm	MRKEHTDNKDLNRTLFLITFAGITPLIIIFITYTSNPKLYLISIIFDNTQNIPSIISAYNPVMTKVMDIYGKSAPLLAIAFTLQLRDR
Colicin N imm	
Marcescin A imm	MR VN IN MYHEIEEGNN IAKKMLCLLLVALVPLLLIAALYY IN PKSNFLQGVS EYTT FLPA IVS SNN PLFS KVMDIYLKTS PLFS FFFFSFYKRI
Colicin Ib imm	MKLDISVKYLLKSLIPILILTVFYLGWKDN
Colicin Ia imm	MNR KYY FNNMWGWVTGGYML YMS WDY
Alveicin A imm	MTEKRTKMLTLWVTPDEHE
Alveicin B imm	MTRKYY IHNM FWG YFMA VCILYAS YGD
Colicin 10 imm	MTVKYYLHNLLE
Colicin K imm	
Colicin 5 imm	
Colicin El imm	MSLRYY IKNI LFGLYCTIY IYLI TKNS
Colicin A imm	KLINNTDRNTVLRSCLLSPLVYAAIVYLFCFRNFELTTAGRPVRLMATNDATLLLFYIGLYSIIFFTTYITLFTPVTAFKLLKKRQ
Caliaia II imm	27 BY CTARGENT THE ATTRACT WATER AND A VERY AND A REACTION OF THE PRODUCT AND A DESCRIPTION OF

Colicin A imm KLINTERNTV---DESCLISSEVYAAIVIFOFENFELTAGGEVKIMASENDILLEYIGLYSIIFFTTYITLEFTVTAFKLIKKAQ Colicin U imm KLIKAINREKL--ITACLITEFIYFYAYFEIANNEELTAGGEVKIMASENDITLEFIGLYSIFFTTYITLEFTVTAFKLIKKAQ Colicin B imm EIIKPTTRLV--VQSCWGUVYAILIYITFINLEITAGGEFKLISHNITUFIYCSIYFTVITTYAILLMELUVIKYFKGRQ Colicin S imm KLIKKINENAV--USSCWGUVYAILIYITFINLEITAGGEFKLISHNITUFIYCSIYFTVITTYAILLMELUVIKYFKGRQ Colicin N imm KLIKKINENAV--LASCLISFFACAVEIVILOFENLEITAGGEFKLISHNITUFIYAILUSSIYFTVITTYAILLMELUVIKYFKGRQ Colicin N imm ELKFKSSPLRA--LKILTFFTILYISHIYTFICTAGEFKLISHNITUFI Marcescin A imm KLKSNGSVSKL--UVFFIFTIYYVCLIVGFFTNIELINSKTFLKAMSTNDITUFIYFVITYALOYIPVYXKILKEB Colicin D imm OFKAMEYYAFICOIISAITFFFSMINIOGULTDTEITSSKTFVLIVKRSVPVFFLYNTIYAILVFYKIKIKSF Colicin A imm KLKSNGSVSKL--UVFFIFTIYYVCLIVGFFTNIELINSKTFLKAMSTNDITEFIFVITYFKLIKSGVAVITVFILALSGK Colicin A imm KLKSNGSVSKL--UVFFIFTXFYVCLIVGFFTNIELINSKTFLKAMSTNDITEFIFVITYSLIVFSITVITVGCLYLKFEGGEV Alveicin A imm FKYRLFRUIS--LCGMUNVAKWYIEDPALKFFRFDWINSGFADFFCMVIGNININGIAVYTGVFILSIFLSHTYLISVITKALSGK Alveicin B imm FKYRLFRUIS--LCGMUNVAKWYIEDPALKFFRFDWINSGFADFFCMVIKININGIAVYTGVVALLTLEFELGPLEGOLFEGOR Alveicin B imm EFKILALRIFG--LASAILFFSFRLIEKTAFTKKEWENGFFTNINGISKILSVILVFILVIKKSPVYVAVILFIGVISFFTNAKL Colicin D imm LIPHUSLILL--VKYPFSLIFISSINUIYYSKUTIS-SINTYKKKERPYSVLEYFLINANIPVGLSFJTYSLKRN Colicin M LIPHUSLLIK--NDNTPLLFFISSINUIYYSKUTIS-SINTYKKKERPYSVLEYFLINAIPVGLSFJTYYLKRN Colicin 5 imm LIPHUILLTH--DNNTPLLFFISSINUIYYSKUTIS-FYLKENTKLKKERPYSULEYFLINAIPVGLSFJTYTLKRN Colicin 5 imm LIPHUILLTH-DNNTPLLFFISSINUIYYSKUTIS-FYLKENTKLKKEPYYLCKISALEYLLAMIPVGLSFJTYTLKRN

Fig. 3.5 Continued

Table 3.1 Pairwise	e distance of DNa	tse killing domain	is and associated i	immunity protein	us from colicins ar	ıd colicin-like ba	acteriocins	
	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 dom	nains (percent sin	nilarity)						
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 immun	uity proteins (per	cent 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	* * *

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1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	Statice of too through to	in nun aunnun guun	sociated minimum pr			
	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 do	mains (percent simila	ritv)				
Cloacin DF13	T.	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 dive	ergence)				
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	***

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



Fig. 3.6 Maximum-likelihood phylogram of nuclease killing domains of colicins and colicinlike 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

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domains which are freely exchanged between colicins and CLBs. Thus, killing domains appear to be transferred more frequently than the targetdefining (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 LexAbinding 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,

-35	5 promoter -10	promoter	LexA binding box(es)
Colicin El Colicin E2 Colicin E3 Colicin E7 Colicin F7 Colicin B Colicin B Colicin B Colicin M Colicin S4 Colicin S4 Colicin S4 Colicin S4 Colicin S4 Colicin S4 Colicin S4 Colicin S4 Colicin C Rebicin C Riebicin C Riebicin C Colicin T Colicin T Colicin T Colicin C Stebicin C Colicin C Stebicin C Colicin T Colicin T Colicin T		TA CC TT TT A T G CT TA CC TT TT G AT GT TA CT TT TT G AT GT TA CT TT TT G AT GT TA CT TT TT G AT GT TA GT TT TT G AT GT TA GT TT G T G CT TA GT TT G T A GT TA CT TT G T A GT TA CT TT A A GT TA CT TT G		A GT A TTI AT TTI TT
Alebian B Colicin A Colicin E Colicin E Colicin E Colicin E Colicin 5 Colicin 5 Colicin 5 Colicin 5 Colicin 5 Colicin 5 Colicin M Colicin M Colicin 94 Colicin 94 Colicin 94 Colicin 74 Alveicin A Alveicin D Klebicin C Klebicin C Klebicin D Klebicin D Klebicin B Colicin I Colicin I Stepicin B Colicin I Colicin D Klebicin B Colicin I Colicin I Colicin D			201 100 <td>MARCH THEORY CLOCK OF A A MARCH CLAR THAC MARCH THEORY CLOCK OF A CANAGE CLAR THAC MARCH THEORY CLOCK OF A CANAGE CLAR THAC MARCH THEORY CLOCK OF A CANAGE CLAR THAC MARCH THEORY CLARANCE CLAR THAT THE THAC MARCH THEORY CLARANCE CLAR</td>	MARCH THEORY CLOCK OF A A MARCH CLAR THAC MARCH THEORY CLOCK OF A CANAGE CLAR THAC MARCH THEORY CLOCK OF A CANAGE CLAR THAC MARCH THEORY CLOCK OF A CANAGE CLAR THAC MARCH THEORY CLARANCE CLAR THAT THE THAC MARCH THEORY CLARANCE CLAR

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.

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