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Prokaryotic Antimicrobial Peptides

From Genes to Applications

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Part I
Introduction: History, Current Knowledge, and Future Research on Antimicrobial Peptides

Chapter 1
History, Current Knowledge, and Future Directions on Bacteriocin Research in Lactic Acid Bacteria

Ingolf F. Nes

All organisms, both eukaryotic organisms and bacteria, are able to produce ribosomally antimicrobial peptides. In bacteria, such compounds are referred to as bacteriocins. The history of bacteriocins goes back to the early 1920s. One has experienced many disappointments in the efforts how to put these compounds into practical use despite being one of the most promising groups of antimicrobial agents to fight bacterial pathogens. However, today, we see new possibilities how to take advantage of such peptides for the benefit of man and animals. Bacteriocin production has become an important property of probiotic bacteria, and targeted use of bacteriocins to fight certain pathogens may have a future.

We should separate bacteriocins from our traditional peptide antibiotics. First, the peptide antibiotics differ from ribosomally synthesized peptides because peptide antibiotics are synthesized by enzymes. Second, bacteriocins are targeted at a narrow spectrum of bacteria often within the species of the producer or closely related ones, while the classical antibiotics are active against broad spectra of bacteria. Another feature that separates bacteriocins from antibiotics is their potency against susceptible bacteria; bacteriocins are unique because they can kill bacteria at nanomolar concentrations, while antibiotics are needed in much higher concentrations.

For many reasons, it is meaningful to separate the bacteriocins of gram-positive and gram-negative bacteria, and this short overview focuses on bacteriocins from gram-positive bacteria. It is most fruitful to divide G+ bacteriocins into two major groups: the heat-stable lantibiotics (Class I) and the nonmodified (some minor modifications may exist) and heat-stable bacteriocins (Class II). These two major classes are further divided into subclasses (Chatterjee et al. 2005; Cotter et al. 2005; Nes et al. 2006). Numerous excellent reviews on bacteriocins have been published

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in recent years (Breukink and de Kruijff 2006; Chatterjee et al. 2005; Cotter et al. 2005; Diep et al. 2009; Drider et al. 2006; Nes et al. 2007; Nissen-Meyer et al. 2009; Oppegard et al. 2007; Willey and van der Donk 2007).

The focus of bacteriocins in gram-positive bacteria has for the most part been on lactic acid bacteria (LAB) due to their apparent importance in food and feed fermentation, and also by being considered as GRAS organisms by FDA, and not least because of good funding in the 1990s and into the twenty-first century by the European Union. An important reason for research on bacteriocin has been and still is their extreme potency as antimicrobials as observed with some bacteriocins that are active at nanomolar concentrations against a number of bacteria including pathogens such as *Listeria monocytogenes*. Some bacteriocins exhibit activity against multidrug-resistant nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE). Thus, it is believed that they could have a potential in medical and veterinary applications.

Fermented food and feed and plant material have been a source for isolation of bacteriocin-producing LAB, but intestinal and fecal sampling of LAB from animals and humans has become an increasingly important source for such bacteria due to an increased awareness of their importance as probiotic bacteria.

Modification and Structure of Bacteriocins

Lantibiotics are gene-encoded peptides that contain intramolecular ring structures by the formation the thioether bridges between dehydrated serine or threonine and cysteines that confer lanthionine and methyllanthionine residues, respectively. Additional but less frequent modifications have been identified in some lantibiotics. Such modifications include lysinoalanine, 2-oxybutyrate, 2-oxopropionate, 2-hydroxypropionate, β-hydroxy-aspartate, S-aminovinyl-D-cysteine, S-aminovinyl-D-methylcysteine, and D-alanine.

After the discovery of lantibiotic bacteriocins that goes back to the early 1920s, it took more than 50 years for the structure of nisin, the first identified lantibiotic, to be determined, i.e., in 1971 (de Klerk and Smit 1967; Gross and Morell 1971). In the 1960s, it was reported that lactobacilli produced antimicrobial substances different from the organic acids (de Klerk and Smit 1967; Sabine 1963; Tramer 1966). It was a slow start, and the area of lantibiotic research did not take off before the 1970s. Since then, numerous lantibiotics have been identified and characterized with respect to structure, mode of action, genetics, regulation, synthesis, and modification. Many excellent and comprehensive review articles have been published in recent years on these topics of lantibiotics (Bonelli et al. 2006; Chatterjee et al. 2005; Dufour et al. 2007; Guder et al. 2000; Pag and Sahl 2002; Twomey et al. 2002; Willey and van der Donk 2007; Xie and van der Donk 2004).

There is presently focus on development of bioengineered lantibiotics, to reveal the location of essential and variable domains therein and to create derivatives with

broader specificity, increased stability, and even higher activities against specific target organisms for both in vivo and in vitro use.

In vitro modification systems have successfully been used to introduce thioether rings into other biologically active peptides. These enzymes have been the focus of recent bioengineering studies.

Of particular significance with respect to posttranlational modification is the new bacteriocin thuricin CD, a strong anticlostridial bacteriocin that is particularly effective against *Clostridium difficile* (Rea et al. 2010), produced by *Bacillus thuringiensis*. This two-peptide bacteriocin is quite unique not just because of its anticlostridial activity but also because both peptides feature three posttranslationally modified sulfur to alpha carbon in thioether linkages. It should also be added that such modification has previously been identified in the circular bacteriocin.

Also, class II bacteriocins can be structured by inducing certain posttranslational changes, and the most common modification is the conserved N-terminal cysteine-bridge formation that has shown to be of crucial importance for the antimicrobial activity of class IIa bacteriocin (Eijsink et al. 1998).

Also, circular bacteriocins are posttranslationally modified through a head-to-tail backbone covalent linkage (Maqueda et al. 2008).

Structures of many bacteriocins have been resolved by NMR analysis. Such studies include both classes of lantibiotics, Class II bacteriocins (Kristiansen et al. 2005; Oppegard et al. 2007; Rogne et al. 2009; Sprules et al. 2004) and cyclic bacteriocins (Gonzalez et al. 2000; Martin-Visscher et al. 2009). Combined with functional analysis, important structural features important for the antimicrobial activity have been determined. In addition, immunity proteins have also been structurally determined (Johnsen et al. 2005; Martin-Visscher et al. 2008). Hopefully, these structural studies combined with functional studies will bring together how the bacteriocins work and how the immunity interacts with its bacteriocin to prevent self-destruction of the host.

Genetics

The genes required for biosynthetic machinery of lantibiotics are complex and are often organized in operons. Together with the structural gene(s) (*lanA*), genes encoding modification enzymes, externalization system of the bacteriocins as well as immunity genes to protect the producer for self-destruction are needed. In addition, it has been shown that the production of some lantibiotics is also regulated by a two-component regulatory system (Kleerebezem et al. 2001), although alternative regulatory systems are identified in a few lantibiotics as seen for lactocin S (Rawlinson et al. 2005).

The modifications are introduced either by one biosynthetic enzyme (LanM) or by a dehydratase (LanB) in combination with a cyclase (LanC). The structure of NisC has been resolved; the reaction mechanism of LctM has been studied, and the amino-acid residues in the active site were identified by mutagenesis studies

(Chatterjee et al. 2006; Li and van der Donk 2007; Li et al. 2006; Rink et al. 2007; You and van der Donk 2007).

Class II bacteriocins need usually only four genes for synthesis that constitute a structural bacteriocin gene, a dedicated immunity gene, and a transporter and its accessory gene.

As seen with some lantibiotics, class II bacteriocins can also be regulated by a two- or three-component regulatory system, and under such circumstances, three more genes are needed: a gene that encodes a peptide pheromone, and genes for a sensor (histidine protein kinase) and a DNA-binding protein that activates gene expression (response regulator) (Nes et al. 1996; Nes and Eijsink 1999). Often, several bacteriocins are clustered together and share both the transport and regulatory system as seen in the plantaricin system (Diep et al. 2009).

Mode of Action and Targets

Numerous mode-of-action studies have been published through all these years, and the membrane has been the target for most of these studies. With few exceptions, bacteriocins cause membrane permeabilization, triggering leakage of intracellular compounds and dissipation of the proton-motive force. Such studies have been carried out with both whole cells and various liposome and vesicle systems. Most of the permeabilization studies suffered by using higher concentrations of bacteriocins than what is needed to kill living target cells. The most obvious conclusion from such studies is that the observed leakage of intracellular components was most likely due to detergent effects and other secondary effects produced by high bacteriocin concentrations and was not associated with the in vivo mode of action.

The identification of lipid II as target of nisin was of crucial importance to understand the antimicrobial activity of this bacteriocin (Brotz et al. 1998). It was a breakthrough when it was shown that nisin actually binds to lipid (Breukink and de Kruijff 2006; Brotz et al. 1998; Pag and Sahl 2002; Schneider and Sahl 2010). Recently, it has been shown that lipid II is the target not only for certain lantibiotics but also for plectasin, a fungal defensin that acts by binding to the bacterial cell-wall precursor (Schneider et al. 2010).

Some class II bacteriocins have been shown to need a specific target for activity. Already in the early 1990s, it was suggested that lactococcin A, a class II bacteriocin, kills susceptible bacteria through a target. This suggestion was based on a study that proposed a membrane-associated protein specific for lactococci acts as receptor for lactococcin A (van Belkum et al. 1991). But this notion was not proved before 2007, when it was experimentally demonstrated that a membrane protein component of the man-PTS system was the actual target site of lactococcin A (Diep et al. 2007). Also, class IIa bacteriocins target the same membrane component of the mannose PTS system, but the recognition specificity is different from that of lactococcin A (Arous et al. 2004; Diep et al. 2007; Gravesen et al. 2002; Héchard et al. 2001; Kjos et al. 2009). It has now been shown that amino-acid sequence of

ManC membrane protein is required for the target specificity of the class IIa bacteriocins (Kjos and Diep unpublished results).

Not only bacteriocins from gram-positive bacteria are using the mannose-PTS as a target for killing but it has been demonstrated that certain microcins from gram-negative bacteria also need this membrane protein(s). A study of microcin E492 produced by *Klebsiella pneumoniae* has shown that a mannose permease is involved in the antimicrobial activity, too. The work concluded that the toxicity is strictly dependent on the presence of ManYZ, the inner membrane protein complex involved in mannose uptake (Bieler et al. 2006).

In the future, we will certainly disclose new targets for other bacteriocins, and there are good reasons to believe that all bacteriocins do have specific targets/receptors/docking molecule.

Application

Many foodborne lactic acid bacteria produce bacteriocins, and several of the most recognized bacteriocins have been isolated from such LAB obtained from fermented food, just to mention, nisin, lactococcin A, and lacticin 3147. Therefore, we can conclude that bacteriocins have a long history of use in food production, on one hand. On the other hand, implementation of either bacteriocin-producing starter cultures or use of cell-free bacteriocin supplements in foods has been much less. But both kinds of products are available for commercial applications. It is surprising that the industry has not used this opportunity more, but this may be due to too high expectation of such compounds that set back such efforts. Several attractive applications have been investigated, but only a few are presently in use.

In recent years, probiotics have become very popular, and LAB are important probiotic players among them. Bacteriocin production has always been considered a beneficial and probiotic feature of LAB, but it has been questioned if bacteriocins are produced and work within the intestinal tract of the host. In a recent work, it has been shown that bacteriocin-producing *Lactobacillus salivarius* protected mice against infection with the invasive foodborne pathogen *L. monocytogenes* (Corr et al. 2007). It was conclusively confirmed that bacteriocin production was the primary mediator of protection against *Listeria* infection. This study supports the idea that bacteriocin production should be included as an important trait and a criterion for selection of probiotic bacteria.

Medical application of antimicrobial peptides, particularly peptides from eukaryotic organisms, has been under investigation for a long time. Bacteriocins have not attracted the same attention mainly because their activities are more bacterial species-specific than that of their eukaryotic counterparts, which often act on both gram-positive and gram-negative bacteria (Zasloff 2002). However, bacteriocins are much more potent against the ones they strike, and they act at nanomolar concentrations, while eukaryotic peptides have to be used at micromolar range.

In order to benefit the broad specificity of the eukaryotic peptides with the high potency of bacteriocins, it may be useful to combine these two activities. It has been demonstrated that by combining pediocin-like bacteriocins (Class IIa) with eukaryotic peptides, a synergistic effect can be obtained (Luders et al. 2003).

Future Trends

There has been a continuous effort to isolate new bacteriocins. The most common approach is to isolate new bacteria, test for antimicrobial activity and then isolate and characterize the activity. This approach is limited by the susceptibility of the indicators used. Also, the growth conditions used for screening new isolates are limiting factors to uncover potential bacteriocin producers, since bacteriocin production can be under the control of a quorum-sensing mechanism (Nes et al. 1996) and the expression can also be off or on only under very narrowly defined conditions (Diep et al. 2000). By the advent of genome sequencing, an increasing number of bacterial genomes are published, and now more than 1,000 genomes are available. Owing to reduced cost of genome sequencing, genome sequencing will be the preferred approach to obtain new bacteriocins and their genes. Development of in silico approaches to identify bacteriocin operons is becoming available, including bacteriocin databases (de Jong et al. 2006, 2010; Hammami et al. 2010). By using such tools, new bacteriocins have already been identified (Begley et al. 2009; Diep et al. 2006; Lawton et al. 2007).

New features will be found and included to improve algorithms for identification of new bacteriocin genes in genome DNA sequence data banks. Recently, a new group of membrane-bound bacteriocin immunity proteins has been identified, and this information has been used to search for novel bacteriocins in sequenced genomes, and seven new bacteriocin-like loci have been identified in gram-positive bacteria (Kjos et al. 2010).

Target identification is a promising area of bacteriocin research. Identification of bacteriocin targets will permit a more detailed molecular analysis and insight into the mechanism of action and rational designs to improve and produce antimicrobial peptides with a broader target specificity and high potency. It is of particular interest that mannose-PTS is target for bacteriocins from both gram-negative and gram-positive bacteria because such PTS systems are unique to bacteria and accordingly bacteriocins should not affect eukaryotic cells. It is tempting to refer to Dr. Erni's commentarial note "Development of The Mannose Transporter Complex: an Open Door for the Macromolecular Invasion of Bacteria" (Erni 2006). His view has been further supported and extended by the finding that the same transport system is the target for Class II bacteriocins in gram-positive bacteria, too.

Peptide and gene sequence information of bacteriocins have laid ground for protein engineering aimed at production of peptides with new properties. Lantibiotics are structurally more complex than Class II bacteriocins and have attracted most focus for protein engineering (Kuipers et al. 1996). Such studies

have also included changes of the dehydrated amino acid as well as lanthionines (thioether bridge) in new peptides. New molecules with increased solubility in water (Rollema et al. 1995) and increased stability and sporicidal activity have been achieved (Liu and Hansen 1992). Hopefully, the future will bring forward new and more efficient antimicrobial peptides based on our knowledge of biologically produced peptides. The future looks bright for both medical and food applications of bacteriocins.

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Chapter 2
Bacteriocin-Mediated Competitive Interactions of Bacterial Populations and Communities

Margaret A. Riley

Abstract Explaining the coexistence of competing species is a major challenge in community ecology. In bacterial systems, competition is often driven by the production of bacteriocins; narrow spectrum proteinaceous toxins that serve to kill closely related species providing the producer better access to limited resources. Bacteriocin producers have been shown to competitively exclude sensitive, non-producing strains. However, the interaction dynamics between bacteriocin producers, each lethal to its competitor, are largely unknown. Several recent studies have revealed some of the complexity of these interactions, employing a suite of in vitro, in vivo, and in silico bacterial model systems. This chapter describes the current state of knowledge regarding the population and community ecology of this potent family of toxins.

Introduction

Bacteria engage in a never-ending arms race in which they compete for limited resources and niche space. The outcome of this intense interaction is the evolution of a diverse and powerful arsenal of biological weapons. Most species of bacteria produce one, and usually many more, of these potent biocontrol agents, including classical antibiotics, lytic agents, lysozymes, and bacteriocins (Cascales et al. 2007). The microbial weapons of choice, as assessed by the frequency with which they are encountered in natural populations of bacteria and in their diversity of forms, are the bacteriocins.

Bacteriocins are loosely defined as biologically active protein moieties with a bacteriocidal mode of action (Tagg et al. 1976; James et al. 1991). Two main features distinguish the majority of bacteriocins from classical antibiotics: bacteriocins are

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ribosomally synthesized and have a relatively narrow killing spectrum (Riley and Wertz 2002). Indeed, bacteriocins are often only toxic to bacteria closely related to the producing strain. The bacteriocin family includes a diversity of proteins in terms of size, microbial target, mode of action, release, and immunity mechanisms and can be divided into two main groups: those produced by Gram-negative and Gram-positive bacteria (Gordon et al. 2006; Heng et al. 2007).

Their production occurs across all major groups of Eubacteria and the Archaebacteria (Webster 1991). Within a species, tens or even hundreds of different kinds of bacteriocins are produced (James et al. 2002; Riley and Gordon 1992). Colicins, bacteriocins produced by *Escherichia coli*, are found in 30–50% of the strains isolated from human hosts and are often referred to as virulence factors (Riley and Gordon 1992). Much higher levels of bacteriocin production have been found in some Gram-negative bacteria, such as *Pseudomonas aeruginosa*, in which >90% produce bacteriocins (Michel-Briand and Baysse 2002).

Despite high levels of bacteriocin diversity, these proteins share many general characteristics (James et al. 2002; De Vuyst et al. 1994). They are generally high-molecular weight protein antibiotics that kill closely related strains or species. The bacteriocin gains entry into the target cell by recognizing specific cell surface receptors and then kills the cell by forming ion-permeable channels in the cytoplasmic membrane, by nonspecific degradation of cellular DNA, by inhibition of protein synthesis through the specific cleavage of 16s rRNA, or by cell lysis.

Without question, bacteriocins serve some function in microbial communities. This statement follows from the detection of bacteriocin production in all surveyed lineages of prokaryotes. Equally compelling is the inference of strong positive selection acting on some bacteriocins (Tan and Riley 1996; Riley 1998). Finally, there is a well-developed body of theory and empirical data that details the potential role of bacteriocins which play in maintaining microbial diversity at the population and community levels (Chao and Levin 1981; Frank 1994; Gordon and Riley 1999; Czárán et al. 2002; Kerr et al. 2002). Such empirical observations and theoretical results argue that these toxins play a critical role in mediating microbial interactions. What remains in question is what, precisely, that ecological role is. Bacteriocins may serve as anticompetitors enabling the invasion of a strain or a species into an established microbial population or community. They may also play a defensive role and act to prohibit the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells. An additional role has recently been proposed for bacteriocins produced by Gram-positive bacteria, that of regulating quorum sensing (Miller and Bassler 2001). This chapter describes the current state of knowledge regarding the population and community ecology of this potent family of toxins.

Colicins: The Model Bacteriocin

The most extensively studied bacteriocins are the colicins, which are produced by *E. coli* (Pugsley 1985; Pugsley and Oudega 1987; James et al. 2002; Cascales et al. 2007). Colicins were first identified almost 100 years ago as a heat-labile product

present in cultures of *E. coli* V and toxic to *E. coli* S. They were given the name of colicin to identify the producing species (Gratia 1925). Fredericq demonstrated that colicins were proteins and that they had a limited range of activity due to the presence or absence of specific receptors on the surface of sensitive cells (Fredericq and Levine 1947).

Colicins are archetypical of a large subfamily of bacteriocins found primarily in the family *Enterobacteriaceae*. One of the defining features of colicin-like toxins is that they are composed of three functional domains: a central binding domain that recognizes and adheres to specific receptor sites on the surfaces of target cells, an amino-terminal translocation domain responsible for entry into the cell, and a carboxy-terminal killing domain that actually kills the cell. Colicins kill target cells through a variety of mechanisms. Nomura showed that colicins E1 and K inhibit macromolecular synthesis without the arrest of respiration, colicin E2 causes DNA breakdown, and colicin E3 stops protein synthesis (Nomura and Witten 1967). In each case, he showed that the lethal action is reversed by treatment with trypsin. Since his pioneering work, colicins were shown to kill their targets by either membrane permeabilization or nucleic acid degradation (Braun et al. 1994; Riley and Wertz 2002; Smarda and Smajs 1998). Colicins are classified according to the nature of the killing domain. The nuclease group includes colicins that degrade DNA, rRNA, or tRNA. The pore former colicins kill by the formation of voltage-gated channels in the cytoplasmic membrane. The third group contains colicins that affect the peptidoglycan cell wall. Colicin operons typically contain three genes: the toxin-encoding gene; an immunity gene, whose product specifically binds to and confers protection against the encoded toxin; and a lysis gene, whose product contributes to the release of toxin into the environment.

Recent surveys of *E. coli*, *Salmonella enterica*, *Hafnia alvei*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* reveal levels of bacteriocin production ranging from 3 to 26% of environmental isolates (Gordon 2006; Riley 2002). Colicins are found in 30–50% of the strains isolated from human hosts (Riley and Gordon 1992). Much higher levels of bacteriocin production have been found in some Gram-negative bacteria, such as *P. aeruginosa*, in which >90% of both environmental and clinical isolates produce bacteriocins (Michel-Briand and Baysse 2002).

Until recently, little was known about the phylogenetic breadth over which bacteriocins kill. To produce such an estimate, we took advantage of a recently determined molecular phylogeny of enteric bacteria (Wertz et al., 2003). The frequency of killing within each taxon for each bacteriocin was mapped onto the enteric phylogeny (Fig. 2.1) (Riley et al. 2003). Not surprising, these data reveal that bacteriocins usually kill members of their own species. However, a surprisingly high level of interspecific killing was observed, with almost half of the bacteriocins killing in more than one taxon. Further, the relationship between killing ability and phylogenetic distance is nonlinear. In other words, some bacteriocin producers kill distantly related bacteria but not their closest relatives. This nonlinear relationship is seen in Fig. 2.1, in which 18 of 36 columns indicate killing outside of the producer strains own species (off the diagonal). The observation of a broad killing range for numerous enteric bacteriocins requires that the ecological role proposed for bacteriocins be reconsidered. It may well be that they serve a broader, community

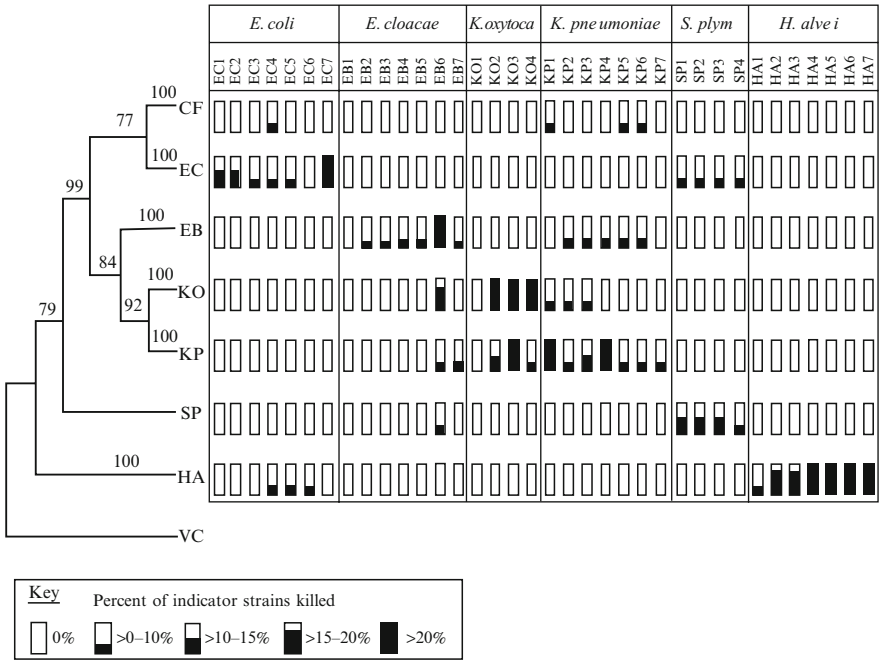

Fig. 2.1 Enteric bacteriocin phylogenetic killing range. The frequency of bacteriocin killing within each of the seven enteric taxa is mapped onto a composite molecular phylogeny of enteric bacteria (adopted from Wertz and Riley 2003). The bacteriocins assayed for killing breadth are indicated across the *top* (EC = *Escherichia coli*, CF = *Citrobacter freundii*, KO = *Klebsiella oxytoca*, KP = *Klebsiella pneumoniae*, EB = *Enteroterobacter cloacae*, HA = *Hafnia alvei*, SP = *Serratia plymuthica*, VC = *Vibrio cholera*). Each column provides the frequency of killing for each bacteriocin assayed against 40 indicator strains for each taxa in the molecular phylogeny

level role than has been envisioned to date. This finding complements recent theoretical work, which suggests that bacteriocins (and other microbial defense systems) may be responsible for maintaining much of the extraordinary diversity of microbes observed in nature (Czárán et al. 2002; Kerr et al. 2002).

The Ecological Role of Colicins

Despite the pervasive role of toxin production in the microbial world, little is known about the ecology of this form of competition. Previous theoretical and empirical studies have suggested that toxin production serves as a strategy to obtain access to nutrients (Chao and Levin 1981; Ivanovska and Hardwick 2005; Riley and Gordon 1992). However, a more recent study testing competitive interactions between toxin producers and sensitive yeast strains under low and high nutrient conditions concluded that toxin producers only out-compete sensitive cells in high