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The ecology and evolution of bacteriocins

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In this review we focus on the ecological and evolutionary forces that determine the frequency and diversity of colicins in *Escherichia coli*. To begin, we describe that this killing phenotype is ubiquitous in *E. coli*, with as many as 50% of the isolates from a population producing colicin toxins, and that each population sampled has its own unique distribution of the more than 20 known colicin types. Next, we explore the dynamics of colicinogeny, which exhibits a typical form of frequency dependence, where the likelihood of successful colicin invasion into a population increases as the initial density of colicinogenic cells increases. We then incorporate thoughts on the evolution of chromosomal resistance to colicins and describe how resistance might influence the dynamics of colicinogen invasion and maintenance and the resulting colicin diversity. The final section deals with a genetic and phylogenetic characterization of colicins and a discussion of the evolutionary mechanisms responsible for generating colicin diversity. In this final section we provide details of the different molecular mechanisms known to play a role in generating colicin diversity, including the two most dominant forces in colicin evolution: recombination and positive, diversifying, selection.

Keywords: bacteriocins; colicins; evolution; ecology; *Escherichia coli*

Bacteria possess numerous mechanisms that enable them to respond to environmental challenges [2,3,32]. Of particular importance are a class of mechanisms that apparently play a role in the competitive interactions between members of a microbial community, through the extracellular release of chemicals that inhibit the growth of other microorganisms. These allopathic substances include: metabolic by-products like ammonia or hydrogen peroxide; the 'classical' antibiotics such as bacitracin and polymyxin B; lysozyme-like bacteriolytic enzymes; and the group of protein antibiotics known as bacteriocins, in which we include the classical colicin-like proteins as well as microcins and lantibiotics [19]. There has been a considerable amount of research concerning these anti-microbial compounds as they provide experimental models for the investigation of a variety of biochemical and physiological processes in bacteria, they serve as potential and realized therapeutic agents, and they are used in the biological control of a number of plant pathogens. This body of work has generated a significant number of general reviews [9,15,20,22,25,33], as well as reviews dealing with more specific topics concerning these anti-microbial agents [5,25,43]. Two areas of research that have not received the same level of attention concern the ecology and evolution of anti-microbial compounds.

In this review we focus on the ecological and evolutionary forces that may determine the frequency and diversity of one class of antimicrobial compounds, the colicin proteins of *Escherichia coli*. We believe that the specific concepts presented provide a useful framework for investigating similar questions regarding other classes of anti-microbial compounds. We begin with a brief portrayal of colicins and the phenomenon of colicinogeny. This is followed by a description of the frequency of colicins and their diversity in bacterial populations. The dynamics of colicinogeny are presented and discussed in relation to how this information might explain the relative frequency of different colicins in bacterial populations. We then briefly describe mechanisms of colicin resistance and how resistance, in turn, might influence the dynamics of colicinogeny and the resulting colicin diversity. The final section deals with a genetic and phylogenetic characterization of colicins and a discussion of the evolutionary mechanisms responsible for generating colicin diversity.

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Colicinogeny

Colicins are by far the best characterized group of bacteriocins. They are produced by, and active against, *E. coli* and other members of the Enterobacteriaceae. These toxic proteins can be classed into four groups depending on how they kill susceptible bacteria. There are those that alter the permeability of the cytoplasmic membrane, non-specifically degrade DNA, cleave 16s ribosomal RNA or inhibit peptidoglycan synthesis resulting in cell lysis [20,30]. Twenty-three different colicins have been described and although they comprise a diverse set of protein functions, they share an intriguing number of features. Under conditions of stress, such as UV irradiation or depletion of nutrients, a fraction of colicinogenic bacteria are induced to produce colicin proteins. This induction is mediated by the SOS system and results in the immediate high level production of both colicin and lysis proteins. The release of colicins is lethal to the producing cell, colicinogenic bacteria are specifically protected against the colicins they produce and colicin gene clusters are plasmid encoded [15,21,25,30,34]. The specific protection against the colicin carried is provided by an immunity protein. This protein is encoded in

the colicin gene cluster, is expressed constitutively and interacts specifically with the C-terminal domain of the colicin protein, rendering it inactive.

Although some colicins are produced by more than one bacterial species [33], it is generally assumed that colicins are of greater significance in intra- rather than interspecific competitive interactions. This assumption largely results from observations suggesting that a colicin produced by one species is usually effective against a greater proportion of strains within that species than against strains from different species [33]. Further, colicins have been shown to invade populations of sensitive *E. coli* rapidly under a wide variety of growth conditions [Tan and Riley, unpublished; Gordon and Riley, unpublished]. Although colicins can clearly influence intraspecific competition, further work is needed to determine if colicins play a significant role in interspecific competition. Recent studies of bacteriocins in Gram-positive bacteria suggest that the relaxation of the requirement for specific cell surface receptor recognition of these bacteriocins results in a much wider range of activity than is observed for colicins.

Frequency of colicinogeny in bacterial populations

Colicinogeny is a common trait in the Enterobacteriaceae. Typcially, 25–50% of *E. coli* isolates are colicinogenic [15,33,36]. Little is known about the factors that determine the frequency of colicinogeny in natural populations. Several studies suggest that the phenomenon is more common among pathogenic than commensal isolates. However, this may result from clinical isolates being dominated by only a few clones [see 15 and 39 for examples]. Differences in the level of genotypic diversity sampled in these surveys may introduce biases that can only be overcome by more carefully designed sampling programs. Contrasts between commensal and pathogenic populations are further complicated by the fact that the plasmids encoding colicins may also encode virulence traits. Such is the case for the plasmid encoding microcin V and is thought to explain the high frequency of microcin V in some populations of pathogenic *E. coli* [15,45]. There is also evidence suggesting that colicinogeny may be more prevalent among human isolates than animal isolates. For example, Riley and Gordon [36] found that in the *E. coli* reference collection (ECOR), 50% of the strains isolated from humans produced colicins in contrast to only 16% of animal isolates.

The *E. coli* population of a human gastro-intestinal tract is composed of numerous clones (genotypes). Some of these clones appear to be resident in that they can be repeatedly recovered over considerable periods of time, while others are transitory and are present for only a few days [4]. Although this dichotomy is in part due to the waxing and waning of the population densities of different clones within the gut, there is no doubt that these observations are also due to the invasion of new clones from external sources. Does colicinogeny either enhance establishment success or enhance defense by resident strains? As is common with much of the work concerning the ecological significance of colicinogeny, the relevant studies have produced contradictory results [8,7,41]. There are interpretational problems associated with most of these studies because it is not possible to determine whether the success or failure of a strain was due to the effects of Col plasmid carriage or due to an unknown fitness effect determined by genes on other plasmids or on the chromosome. However, it is intriguing that several studies have documented the successful invasion of strains in the gut and urinary tract that also produce colicins.

Might the frequency of immigration-extinction events in a host determine the frequency of colicinogeny in *E. coli* populations? Total genotypic diversity (D_T) which is usually assayed with multi-locus enzyme electrophoresis, varies between *E. coli* populations: $D_T = 0.67$ in a single human host [47]; $D_T = 0.85$ in a chicken flock [47]; $D_T = 0.91$ in a feral mouse population (unpublished data). D_T also varies temporally between populations with levels of diversity fluctuating over time by 53% in a single human host; 26% within a chicken flock and 5% within a feral mouse population. These results suggest that there are substantial differences in the turnover rates of clones in different populations and perhaps in different hosts as well. Thus, differences in colicin frequencies in different hosts may simply reflect the different turnover rates of clones in these hosts.

Relative abundance and diversity of colicins

The frequency of different colicin types varies substantially between populations. Some sense of this variation can be seen in Figure 1 which presents the relative frequency of different colicins in four collections of *E. coli* isolates. The number of different colicins identified in each sample varied from three to nine, while the relative frequency of a particular colicin ranged from 0 to 88%. Colicins that are abundant in one sample can be rare or absent in others. The available data does suggest that those colicins carried by self-transmissible plasmids (eg B, Ia, Ib, V) are no more frequent in *E. coli* populations than the colicins encoded for by non-conjugative plasmids. Many clones of *E. coli* have a cosmopolitan distribution and this appears to be true of many of the colicins found in this species as well [1; unpublished data]. Do factors other than chance act to determine the relative abundance of different colicins in bacterial populations?

Population dynamics of colicinogeny

There have been several theoretical investigations concerning the population dynamics of colicinogeny [6,13,24]. These studies have shown that whether a colicin-producing strain successfully invades a population or not depends on the cost versus benefits of colicin possession. The costs associated with colicin production include: 1) the loss to the colicinogenic population due to colicin synthesis and subsequent cell lysis; and 2) the growth rate disadvantage resulting from the energetic costs imposed by colicin plasmid carriage. For the colicin-producing strain to invade, these costs must be exceeded by the advantage gained due to colicin production. The magnitude of this advantage is determined by: 1) the initial density of colicinogenic cells; 2) the rate at which sensitive cells are killed by the colicin,

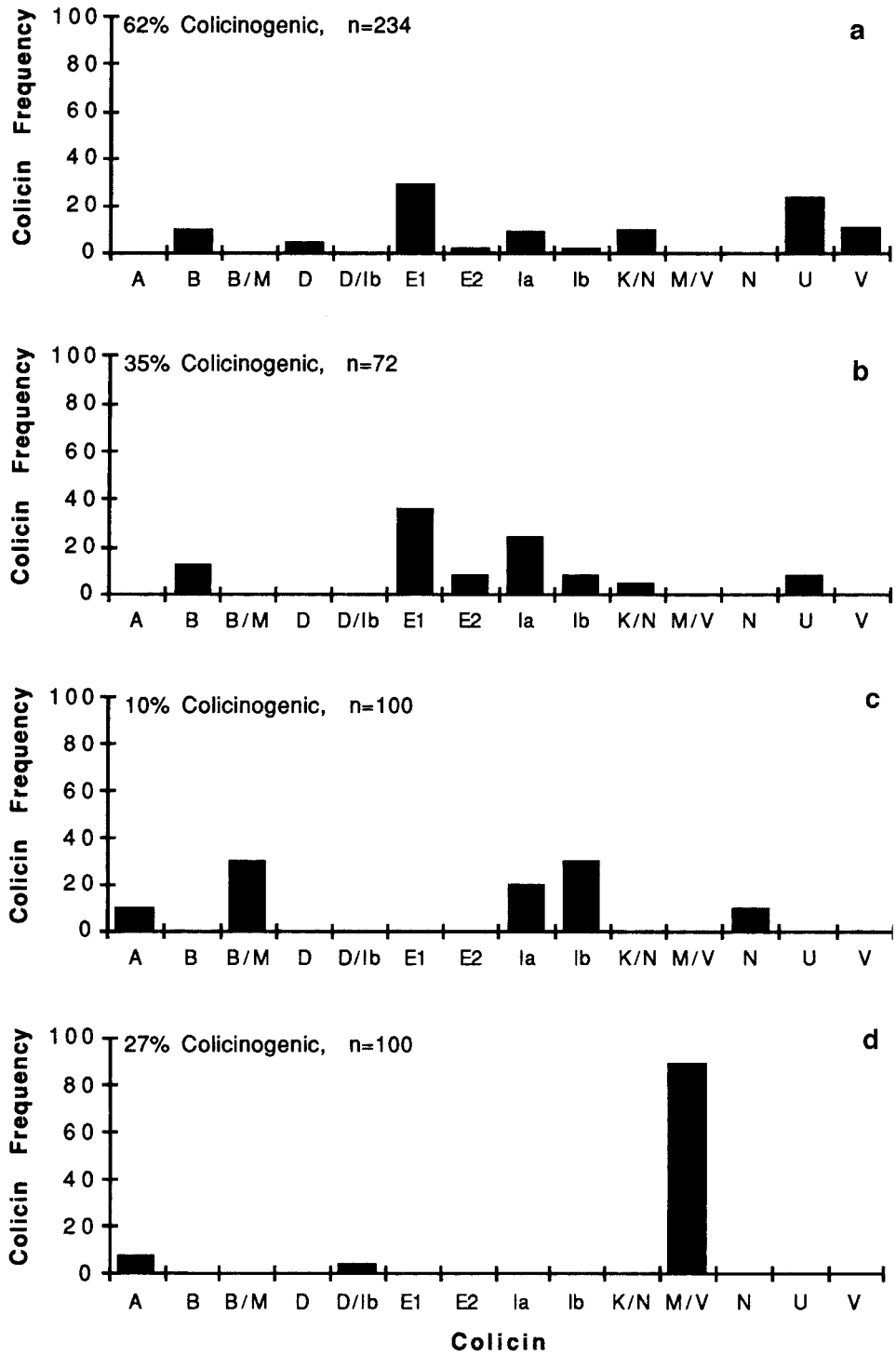

Figure 1 The relative abundance of different colicins in four collections of *E. coli* isolates. (a) Isolates from symptomatic humans [1]. (b) Isolates from humans and animals [36]. (c) Isolates from symptomatic humans (unpublished data). (d) Isolates from chickens (unpublished data).

which is determined by the amount of colicin released per lysed cell; and 3) the rate at which the colicin binds to sensitive cells. Thus the dynamics of colicinogeny is a typical example of frequency-dependence, where the likelihood of successful invasion increases as the initial density of colicinogenic cells increases (Figure 2).

The invasion dynamics of colicinogeny described so far have dealt with liquid habitats [6,24]. Similar outcomes result from consideration of the dynamics of colicinogeny for bacterial populations growing on surface habitats [6,24]. In both cases, frequency dependence favors the more common type, susceptible or producer. Further, higher rates of colicin production enhance the probability of invasion by the colicinogenic strain. There are two main differences in the outcome of competition between liquid and surface habitats. First, the colicinogenic strain can typically invade

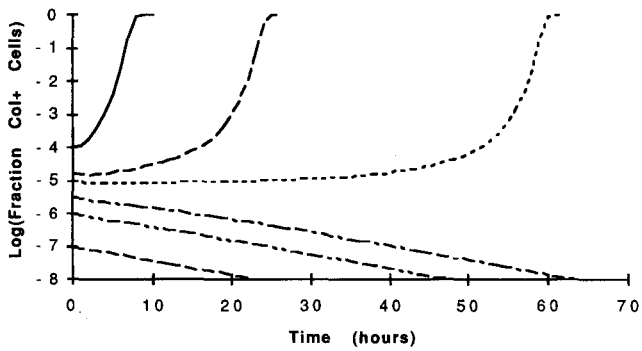

Figure 2 The change in the frequency of colicinogenic cells over time at different initial starting frequencies. The model used represents a variant of these models and describes the invasion of colicin producers in a chemostat system. There are two cell populations, the colicin-sensitive population and the colicin-producing population. Producers lyse at a constant rate, thereby releasing molecules of colicin. Sensitive cells are killed by free colicin at a rate proportional to the product of colicin molecule and sensitive cell densities and the absorption rate parameter. The sensitive cell population grows at a rate ψ_r which is dependent on resource concentration. The colicin-producing population grows at the rate $\psi_r\ (1-\sigma)$ where σ is the selection coefficient. Resource is taken up at a rate proportional to the bacterial growth rates and cell density with a conversion efficiency of ϵ.

a surface habitat at much lower initial frequencies than in a liquid habitat [6], which may be due to the colicin-producing colony being better able to sequester resources on a solid surface. Second, variation in habitat quality (resource concentration) has a greater influence on the outcome of competition on surfaces, with colicin producers being favored more in poor quality habitats, while high quality habitats favor susceptible cells [13]. This latter effect is likely due to the cost of colicin plasmid carriage in high quality habitats where rapid cell growth may be the primary determinant of invasion success.

Colicinogeny dynamics and the abundance of different colicins

Above we showed that there is considerable variation in the relative frequency of different colicins both within and between bacterial populations. Can the dynamics of colicinogeny tell us anything about the frequency at which different colicins occur in bacterial populations? An important determinant of the invasion success of a colicinogenic strain is the rate at which the colicin-producing strain is able to kill sensitive cells. Two factors determine the death rate of sensitive cells: adsorption of the colicin and the number of colicin molecules released per lysed cell.

It is generally assumed that the adsorption of colicin to sensitive cells fits a model of single-hit kinetics, that is, one colicin molecule is sufficient to kill a cell [33]. The rate at which colicin molecules adsorb to a cell will depend on the concentration of colicin molecules and sensitive cell density, but in addition it will also depend on the number of surface receptors present on sensitive cells. Different colicins exploit different cell surface receptors and these receptors are present in very different numbers, ranging from 20 to 2000 per cell [33]. This suggests that there may be significant differences among colicins in the rate at which they adsorb to sensitive cells.

A number of different colicins can exploit the same surface receptor [20]. One such receptor is BtuB which is used by all the E colicins. Among the E colicins the number of sensitive cells killed by the colicin released from a given number of producers varies by more than three orders of magnitude, indicating substantial differences in the number of colicin molecules released per induced cell. This variation has significant consequences for the invasion dynamics of colicinogenic bacteria. In essence the minimum initial frequency required for invasion decreases as the number of colicin molecules released per lysed cell increases (Figure 3). These theoretical considerations suggest that factors such as rate of colicin release per induced cell may be of significance in determining the relative frequency of different colicins.

Resistance to colicins

Colicin molecules bind to specific outer-membrane receptors. In the case of the colicins E1-E9, the BtuB protein, which functions as a receptor for vitamin B_{12} [11,20,44] is employed. Colicin resistance arises when mutations occur that result in either the loss, or alteration, of a receptor. Tolerance to colicins also occurs and is thought to be due to mutations in the system involved in translocating the colicin from the receptor to its target in the inner plasma membrane [16,28,29,46]. Mutations conferring resistance or tolerance to one colicin may also confer decreased susceptibility to other colicins [31]. For example a mutation in the *BtuB* gene causing the loss of the B12 receptor protein can confer resistance to the entire E series of colicins and partial resistance to colicin A. The cost of resistance may be substantial, as can be observed with resistance to the bacteriophage T4 (which also involves the loss of a cell surface receptor), where the relative fitness of resistant mutants may be half that of the susceptible strain [23]. Further evidence of the cost of resistance comes from competition experiments between resistant and sensitive strains of *E. coli*. In each case examined thus far, sensitive cells can rapidly invade resistant populations (Tan and Riley, unpublished; Feldgarden and Riley, unpublished).

Using the *E. coli* reference collection, Riley and Gordon [36] determined the frequency of resistance to a range of colicins and found that the majority of *E. coli* strains were resistant to most colicins. Subsequent studies, using different collections of *E. coli*, have produced similar results

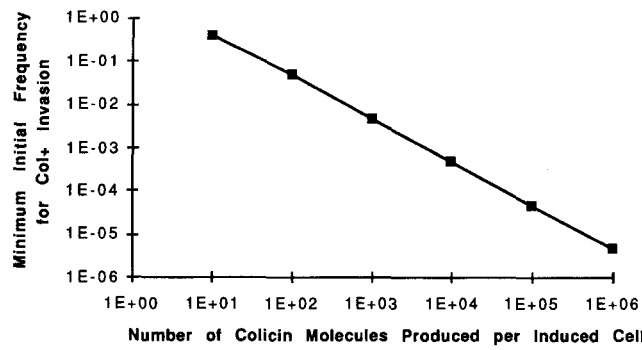

Figure 3 Minimal initial frequency required for invasion as a function of number of colicin molecules produced per lysed cell.

(Feldgarden, Gordon and Riley, unpublished results). Interpetation of these results is complicated due to the fact that the degree of resistance detected in a strain collection depends on the colicinogenic strains used to produce the colicin. For example, less than 2% of ECOR strains are susceptible to the colicins produced by a Col E1 or Col Ib plasmid in the *E. coli* K12 host W3110. However, 60% of the ECOR strains are susceptible to the same colicin when produced by one of the ECOR strains (E1, ECOR 63; Ib, ECOR 61). This appears to be a general result, but one that does not alter the basic conclusion that in a population of *E. coli* most strains are resistant/tolerant to most co-occurring colicins.

The evolution of resistance is likely to be an important factor determining the relative frequency of a particular colicin in a bacterial population. As the frequency of resistance increases in the bacterial population the fitness advantage of colicinogenic cells will decline, although there will be no change in the costs associated with colicinogeny. Such a situation will lead to a decline in the frequency of the colicinogenic population. As the frequency of colicinogenic cells in the population declines so will the fitness advantage of resistant cells. This may in turn lead to an increase in the frequency of susceptible cells. Does the relative balance of these forces tend to result in the stable coexistence of susceptible, colicinogenic and resistant populations? There have been virtually no theoretical or empirical investigations concerning the dynamics of colicinogeny and the evolution of colicin resistance.

Not only will the evolution of resistance determine the relative frequency of a particular colicin, it will also be an important determinant of the relative frequencies of different colicins. As resistance to one colicin increases in frequency, the likelihood of a colicin exploiting a different outer membrane receptor establishing in this population may also increase. Such an outcome represents a classic example of an 'advantage when rare' type of frequency dependence.

Evolution of colicins

Previous sections have described the phenomena of colicinogeny and discussed ecological factors that may act to determine the frequency of colicinogeny in bacterial populations. This section will deal principally with a genetic and phylogenetic characterization of colicins and a discussion of the evolutionary mechanisms responsible for generating colicin diversity.

A feature common to all colicin proteins is their organization into four functional domains [27,30,37]. Information required for movement across the cell membrane is found in the N-terminal region of the protein. The central portion is involved in receptor recognition and interaction, while the C-terminal portion contains both the killing function and sequences that interact with the immunity protein [20]. Colicin proteins display varying levels of sequence similarity across these functional domains, ranging from virtual identity to undetectable. For example, colicins Ia and Ib employ identical modes of entry into the cell and have identical killing functions but differ in immunity specificity [26]. They differ at only 1% of the N-terminal 430 residues whereas in the immunity binding region 64% of the C-terminal residues differ. In contrast, colicins A, B, E1 and N employ a killing function similar to Ia and Ib; however, genetic and biochemical studies indicate that they have different methods of entry into the cell and different immunity proteins. Comparisons of these colicin sequences reveal no detectable (ie greater than 30%) similarity in the N-terminal two-thirds of these colicins and similarity limited to 50% or less in the C-terminal region.

Riley [34,35] distinguished five classes of colicin proteins, based upon protein sequence comparisons. Figure 4 provides a schematic representation of the classes and the regions of sequence similarity within a class. With one exception (colicin B), there is no detectable sequence similarity between colicin classes. Perhaps the most striking result from these phylogenetic studies of colicin proteins is that this class of proteins represents an incredibly heterogeneous ensemble, even though, at a more superficial level, they seem united as a group as they share an intriguing number of genetic features. Whether the sharing of such a remarkably similar genetic organization among colicins or their common carriage on plasmids, or their common modes of induction represent common ancestry or simply an amazing example of convergence onto a similar killing mechanism awaits further DNA and protein sequence comparisons.

These proteins have, nonetheless, proven to contain a wealth of information regarding the molecular mechanisms of protein evolution. First, although the phylogenies inferred for the colicin, lysis and immunity genes broadly agree [34], several significant discrepancies between lysis and colicin trees suggest that recombination has played an important role in generating novel colicin gene clusters. When a phylogeny is inferred for lysis proteins, the lysis protein for colicin DF13 clusters with that of colicin E1, which is in a different colicin class than DF13, as inferred from a colicin-based phylogeny. This discrepancy, and several others [34], suggest recombination events have moved lysis genes between colicin clusters.

The patterns of domain restricted sequence similarity and differences among colicin proteins implicate an even more restricted form of recombination, ie intragenic recombination, in generating novel colicin functions. For example, DNA and protein sequence comparisons suggest that colicin B shares ancestry with members of two other classes of colicins. The N-terminal residues of this colicin are essentially identical to colicin D [37]. This identity is terminated abruptly in the C-terminal region, where no detectable sequence similarity is observed following one particular base pair. Schramm *et al* [38] noted that 52% of the nucleotides in colicins A and B immunity sequences are identical. This similarity extends through a short intergenic region and into the 3′ end of the colicin gene. Finally, Schramm *et al* [38] suggested that a 294-base pair sequence 5′ to colicin B gene is derived from the colicin E1 gene cluster. Thus, colicin B is composed of three segments: the E1-like upstream region, the D-like N-terminal region and the A-like carboxy-terminal region. Several examples of this domain-based shuffling have been reported [21,34].

Colicin, immunity and lysis genes display quite different levels of nucleotide and amino acid sequence divergence

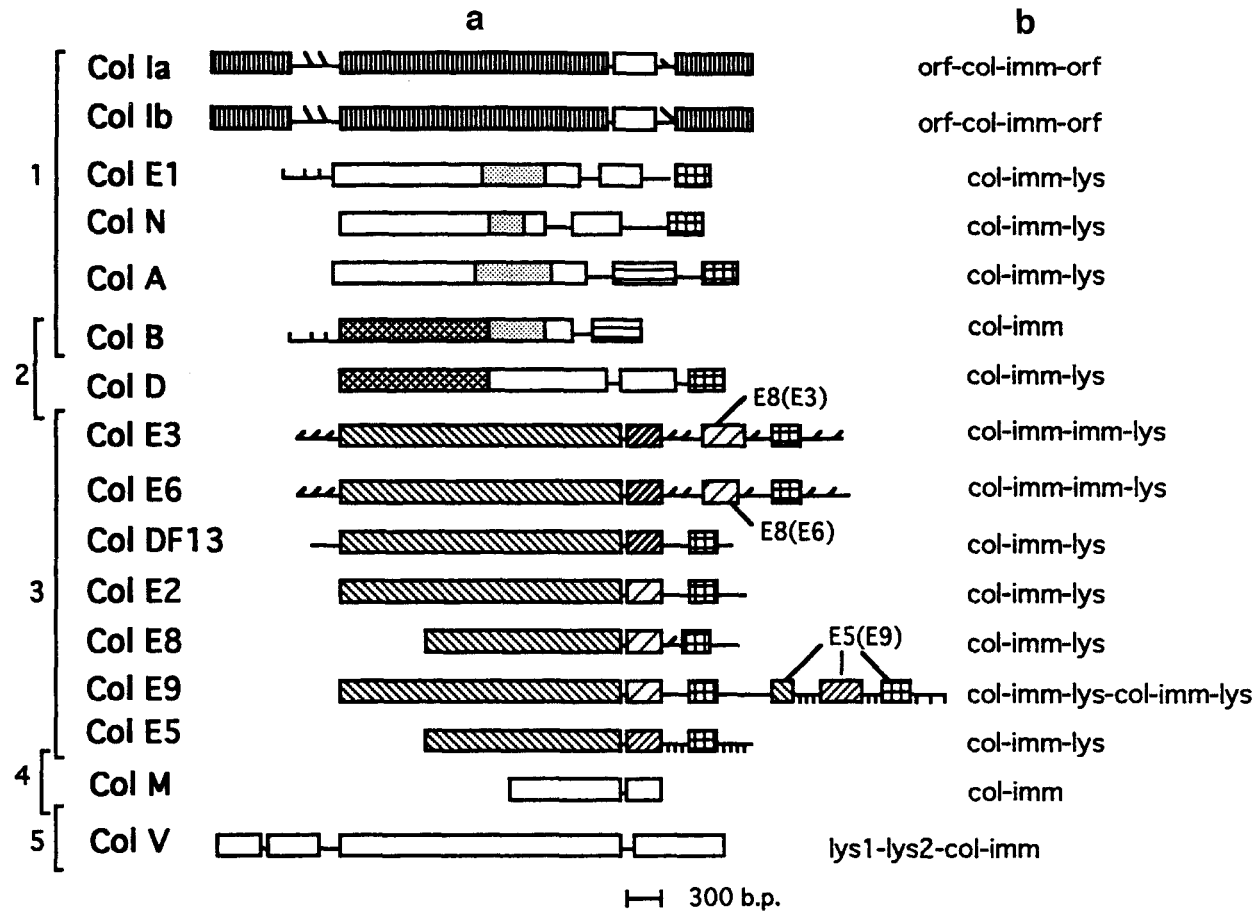

Figure 4 Colicin cluster organization. (a) Colicin clusters drawn to scale. Regions of >80% DNA sequence identity between genes and spacer regions are indicated with matching hatching (angled and vertical lines). Rectangles indicate coding sequences, and connecting lines indicate spacer sequences. Bars on left border and corresponding numbers indicate colicin groups. (b) Gene order for each gene cluster. orf = open reading frame, col = colicin gene, imm = immunity gene, lys = lysis gene. Figure from Riley [34].

[21,34,35]. Colicins Ia and Ib provide the most dramatic example, with almost complete DNA sequence identity across the entire colicin gene cluster with the sole exception being the region that includes the 3′ end of the colicin gene and the immunity gene, where the sequences are essentially saturated for substitutions (Figure 5). An identical pattern of substitution is observed in colicin pairs E3/E6 and E2/E9.

How do we account for these varying levels of substitution across the colicin gene cluster? We can reject the most obvious explanation, ie relaxation of purifying selection in the immunity protein and immunity-binding region of the colicin protein resulting in the accumulation of neutral substitutions, as this hypothesis will not account for the equally high levels of substitution in the silent positions of this region, which presumably would not experience the same selective forces as the encoded protein, and thus should accumulate substitutions at essentially the same rate as synonymous sites in the regions 5′ and 3′ to the immunity region. Further, we can rule out transposition and mutator gene-mediated mechanisms [34,35]. This leaves at least two additional hypotheses. Either recombination has introduced novel regions within these gene clusters, or natural selection is acting differently on this region relative to the remainder of the colicin gene cluster, resulting in the differential accumulation of substitutions. Closer examination of the regions of interest call into doubt the sole action of recombination in producing the elevated levels of divergence. In the cases of colicin pairs E2/E9 and E3/E6, numerous recombination events would be required to explain the pattern of clustered sequence divergence observed in this region. In light of the very limited sequence divergence observed outside the immunity region, it is unlikely that recombination rates are high enough to generate the required number of clustered substitution events within the immunity region during a period when few point mutations have occurred in the flanking regions.

An alternative hypothesis invokes a role for positive selection in generating elevated levels of substitution in the 'immunity region' of the colicin gene cluster. In this scenario, mutations occur that confer broader immunity functions, say, for example, a mutation that causes the host cell carrying the colicin E3 plasmid to be immune to several related E-type colicins. This sort of immunity mutation provides an obvious advantage when multiple colicin types are segregating. Eventually, a mutation occurs in the colicin-binding region which, in tandem with the original immunity mutation, creates a novel colicin type that retains immunity

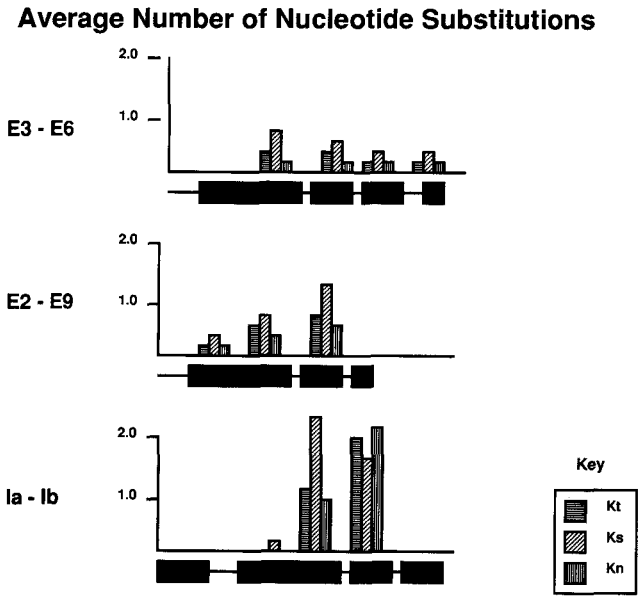

Figure 5 Distribution of nucleotide substitutions along the colicin cluster region for three pairs of colicins. The average number of substitutions, corrected for multiple substitution events, is plotted for the functional domains depicted along the Y-axis. Total, non-synonymous and synonymous sites are considered separately. Figure from Riley [35].

to the ancestral colicin while producing a colicin to which the ancestor is no longer immune. The resulting novel colicin type will rapidly be selected for as it can kill, but not be killed by, the ancestral colicin type.

This form of selection will result in the rapid accumulation of substitutions at non-synonymous sites in both the immunity and immunity-binding regions, as novel colicin types are created and swept into the population. Further, mutations that occur in synonymous sites in this region will accumulate at elevated levels due to a hitchhiking effect; that is, as clusters of mutations in the immunity region are held in the population by positive selection, neutral mutations in the region will accumulate because of their tight linkage to the selected sites [40]. This form of accelerated divergence is analogous to the diversifying selection hypothesized at histocompatibility loci in mammals [17,18].

The pattern of accelerated divergence in the immunity gene and the immunity-binding region of the colicin gene suggests some form of positive selection has played a role in the diversification of colicin clusters. Further, the presence of conserved sequences flanking the regions under selection argues that this process of divergence must occur rapidly. Thus, a combination of frequent recombination events generating novel combinations of colicin functional domains and their subsequent, rapid diversification through the action of positive selection may help to explain the diversity of colicins observed in natural populations of *E. coli*.

This review has suggested a number of approaches for studying the ecology and evolution of anti-microbial compounds. Although the results presented can, at present, only be applied to bacteriocins, and even more specifically, to the subclass of bacteriocins produced by *E. coli*, it is our belief that similar approaches will prove fruitful in attempting to understand how a wide variety of anti-microbial compounds evolve and, perhaps more importantly, how they function in population-level and species-level bacterial dynamics. The importance of this sort of information has only recently begun to be appreciated. Bacteriocins are currently the focus of intensive efforts aimed at designing and implementing biologically-based food preservation methods [10,42]. Further, the potential for employing bacteriocins in clinical, or therapeutic settings, has only just begun to be realized, despite a very long history of interest in such a role [12,14]. Finally, when considered with respect to the release and successful establishment of populations of genetically-engineered microorganisms, bacteriocins and other anti-competitor substances will clearly influence how successfully the released organism interacts with other members of its own species and different species in the environment.

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