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MOLECULAR MECHANISMS OF BACTERIOCIN EVOLUTION

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ABSTRACT

Microorganisms are engaged in a never-ending arms race. One consequence of this intense competition is the diversity of antimicrobial compounds that most species of bacteria produce. Surprisingly, little attention has been paid to the evolution of such extraordinary diversity. One class of antimicrobials, the bacteriocins, has received increasing attention because of the high levels of bacteriocin diversity observed and the use of bacteriocins as preservatives in the food industry and as antibiotics in the human health industry. However, little effort has been focused on evolutionary questions, such as what are the phylogenetic relationships among these toxins, what mechanisms are involved in their evolution, and how do microorganisms respond to such an arsenal of weapons? The focus of this review is to provide a detailed picture of our current understanding of the molecular mechanisms involved in the process of bacteriocin diversification.

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INTRODUCTION

What Are Bacteriocins?

Producing antimicrobial compounds seems to be a generic phenomenon for most, if not all, bacteria. Antimicrobials include toxins, bacteriolytic enzymes, bacteriophages, by-products of primary metabolic pathways, antibiotics, and bacteriocins. One class of antimicrobials, bacteriocins, has received increasing attention because of the surprisingly high levels of bacteriocin diversity observed, the widespread distribution of bacteriocins in bacteria, and the use of bacteriocins as preservatives in the food industry (19) and as antibiotics in the human health industry (42, 52, 92–94).

Bacteriocins are compounds produced by bacteria that inhibit or kill closely related species (50, 104). Their production occurs across all major groups of Eubacteria and the Archaebacteria (100). Some bacteriocins, such as the halocins of Halobacteria, have no obvious protein sequence similarity to any other characterized bacteriocins (88). Others, such as the S pyocins of *Pseudomonas aeruginosa*, some of the colicins of *Escherichia coli*, a cloacin of *Enterobacter cloacae*, a klebicin of *Klebsiella pneumonia*, and a marcesin of *Serratia marcescens*, reveal shared evolutionary ancestries through protein sequence comparisons (75–77, 83, 84, 103; M Riley, unpublished data).

Despite high levels of bacteriocin diversity, these proteins share many of the following characteristics (50, 104). 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 resulting from inhibition of peptidoglycan synthesis. Frequently, the bacteriocin is released from the cell through the action of a lysis protein, although other export mechanisms may be involved. The producing strain shows specific immunity toward its bacteriocin through the production of an immunity protein that usually interacts directly with the C-terminal domain of the bacteriocin protein. The bacteriocin phenotype is usually encoded by a gene cluster of three closely linked, frequently plasmid-encoded, genes: the bacteriocin, immunity, and lysis genes. Finally, the target cell can evolve resistance or tolerance to the effects of bacteriocins, generally by altering a cell surface receptor or translocation system. All of these characteristics are possessed by the well-studied bacteriocins of *E. coli*, the colicins (50, 71, 74). Bacteriocins of gram-positive bacteria and Archaebacteria share some of these characteristics, but can be quite different in their sizes, their modes of killing, their range of effect, and their modes of release and transport in the cell (50, 88, 104). Quite often the bacteriocins of

gram-positive bacteria lack a specific receptor for adsorption, they can be of relatively low molecular weight, and they have leader sequences that are cleaved during maturation (46).

What Is the Function of Bacteriocins?

Several mathematical models have been designed to examine the interaction between bacteriocin-producer and -sensitive strains. Early work suggested that the dynamics of these competitive interactions differ in differing environments (11, 12, 79). Invasion of a producer strain into a sensitive population is predicted to be ineffective in a homogeneous, well-mixed environment, such as liquid culture, unless initial frequencies are high. This is due to the dilution of toxin in liquid culture and the cost of bacteriocin production. Alternatively, it is predicted that low initial frequencies of a sensitive strain will not invade a bacteriocin-producing population in liquid culture due to the high levels of toxin present (11, 12, 79).

However, if there is structure to the environment, such as a solid surface, and there is spatial heterogeneity in resource abundance, then a stable polymorphism of producer and sensitive strains can exist (34). Sensitive strains will persist in poor habitats where the rate of resource competition is high, while producing strains will persist in good habitats where resource competition is low. In this more complex model, the relative abundance of each class of bacteria is determined by initial abundance, levels of competition, migration, and diffusion of toxin (34).

More recent theoretical work has involved the further development of a spatially structured population model in which the outcome of competition is again determined by the effectiveness of killing in relation to its costs and by the incorporation of "cheater" strains that have less costly production and resistance to the bacteriocin of the producer (24). In this more complex set of interactions, it is possible to achieve a dynamic equilibrium between bacteriocin producer, sensitive, and cheater strains.

The models described above focus specifically on the dynamics of colicin-like producing strains. Dykes & Hastings (25) have recently challenged whether models of colicin dynamics can be generalized to other bacteriocins. They raise the argument that different bacteriocins may impose quite different fitness costs (relative to colicins), depending upon whether the bacteriocins are chromosomally or plasmid encoded, whether they function solely as killing agents or have some other function, and what type of environments the producing strain inhabits (25).

Competitive advantages to bacteriocins have been observed directly in laboratory settings with bacteriocin producing *Escherichia coli*, *Vibrio harveyi*, *Enterobacter* spp., *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas*

aeruginosa, *Lactobacillus* spp., and *Myxococcus* spp. (12, 43, 68, 91, 97). Additional studies have examined the action of bacteriocins in more natural settings, such as *Haemophilus influenza* in the nasopharyngeal region of the rat (59), *Streptococcus mutans* in the human oral cavity (41), *E. coli* in the guinea pig conjunctivae and in the rat urinary tract (4, 96), and *Leuconostoc plantarum* in green olive fermentation vats. In each case, the action of the bacteriocin increased the competitive advantage of the producing strain in invading or maintaining populations in a defined setting.

The bacteriocins of lactic acid bacteria, such as nisin, are in use for food preservation. The inhibitory spectrum of these bacteriocins is generally restricted to gram-positive bacteria, but several are active against such food spoilage and food-borne pathogenic microorganisms as *Bacillus cereus*, *Clostridium botulinum*, *C. perfringens*, *L. monocytogenes*, and *Staphylococcus aureus*. Nisin has been recognized as safe in the United States for use in selected pasteurized cheese spreads to prevent spore outgrowth and toxin production by *C. botulinum*, as a preservative to extend shelf life of dairy products, and in spoilage prevention in canned goods (14, 19, 102).

The potential clinical utility of bacteriocins was first suggested in the 1950s. However, the enormous impact of the antibiotic era deterred most research in this area. More recently, interest in bacteriocins as a replacement for traditional antibiotics has increased. Nisin has been used to inhibit plaque-producing bacteria (42). Colicinogenic *E. coli* have been examined for inhibition of *Shigella sonnei* infection of the conjunctivae (96). Competition of colicinogenic and sensitive *E. coli* in the rat urinary tract has been described (4). Colonization of bacteriocin-producing strains of *H. influenza* (in the rat nasopharyngeal region) and *S. mutans* (in the human oral cavity) has been investigated (41, 59). Each of these studies suggests that bacteriocins may play a significant role in the control of bacterial infections.

Results from mathematical and experimental studies, combined with the high frequencies with which bacteriocins are encountered in nature, argue that bacteriocins play a key role in bacterial population dynamics. It is often assumed that bacteriocins act at the level of intraspecific competition. However, none of the studies described above have sought to delineate either the phylogenetic breadth of bacteriocin killing or the specific interactions mediated by bacteriocins in a natural microbial community. At this point, although it is safe to say bacteriocins serve in mediating bacterial interactions, we simply do not have the appropriate data to determine precisely which interactions bacteriocins mediate.

COLICINS AS A MODEL SYSTEM

The colicins of *E. coli* have served as a model system with which to explore the molecular structure and function of bacteriocin and related proteins. We

now have crystal structures for several colicins (including Ia, A, and E1) and for one colicin complexed with its immunity protein (E3) (9, 29, 44, 111, 113). We have a detailed understanding of the genetic organization, regulation, and DNA sequences of over 20 characterized colicin gene clusters. Further, we have detailed models of many of the protein:protein interactions that occur between the toxin, the cell membrane, the immunity protein, and the target (2, 3, 5–10, 21, 22, 30, 35, 50, 51, 54). A particularly intensive effort is currently directed at understanding colicin E9 receptor recognition, modes of translocation, nuclease activity, and immunity binding (7, 16, 17, 27, 48, 49, 66, 105–109).

Colicins have also served as a model system for investigating the mechanisms of bacteriocin evolution and diversification (12, 32, 34, 50, 76–80, 97). Much of this work has involved comparisons of DNA and protein sequences among colicin, immunity and lysis genes, and encoded proteins to infer evolutionary relationships and molecular mechanisms of diversification.

Protein sequence comparisons among colicins reveal two families, the pore former and nuclease colicins (Figure 1), with no detectable sequence similarity observed between the families. Within the pore-former family, levels of pairwise sequence similarity often fall below the "twilight" range of 30% similarity. However, if comparisons are restricted to the C-terminal residues three subfamilies of pore formers emerge: Ia, E1, and A. In contrast, the nuclease colicin family represents a closely related family of proteins. Two subfamilies of nuclease colicins can be distinguished based upon slightly reduced levels

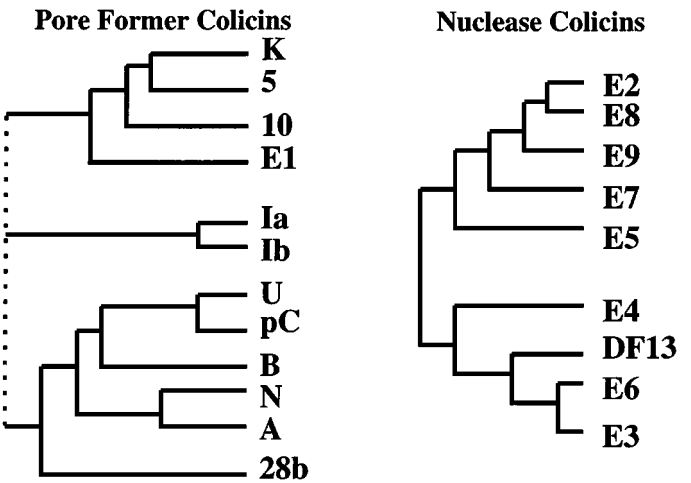

Figure 1 Pore-former and nuclease colicin evolutionary relationships inferred using parsimony methods and the C-terminal 300 amino acids of the colicin proteins.

of protein sequence similarity that, in part, reflect the RNase (E3, E5, E6, E4, DF13) versus DNase (E2, E7, E8, E9) functions of the proteins.

More detailed comparisons of the protein sequences within each of these two families have suggested that they experience quite different evolutionary forces. The following two sections provide a detailed description of the roles of positive selection and recombination in nuclease and pore-former colicin protein diversification. Much of this work was recently reviewed by Tan & Riley (99).

Positive Selection: A Major Force in the Diversification of Nuclease Colicins

The role of positive selection in generating colicin diversity was first proposed to explain an unusual pattern of divergence between two pairs of closely related nuclease colicin gene clusters (colicin pairs E3/E6 and E2/E9) (76, 77). For each pair, both synonymous and nonsynonymous sites in the immunity gene and in the immunity binding portion of the colicin gene (immunity region) are highly substituted (Figure 2).

To explain this excess of clustered substitutions, a two-step process of diversification was proposed (Figure 3). First, a point mutation occurs in the

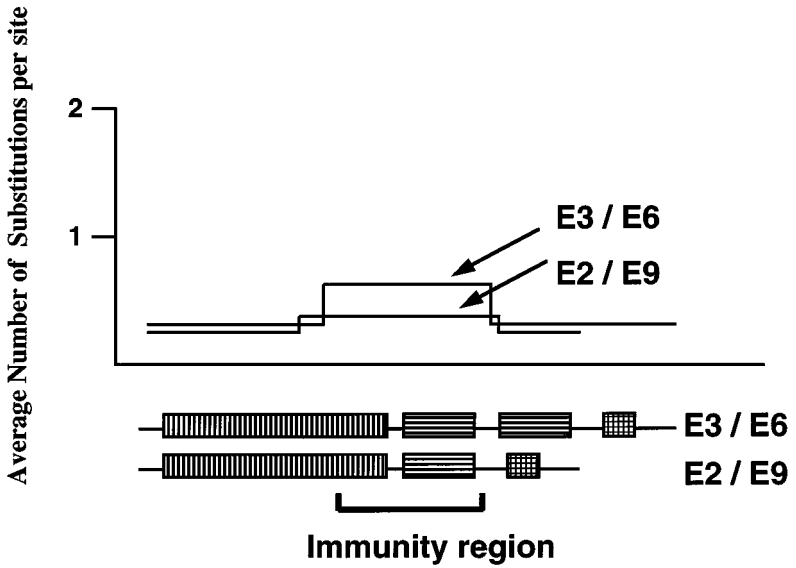

Figure 2 The average number of DNA sequence substitutions between each pair of colicin gene clusters (colicin pairs E2/E9 and E3/E6). The order of the genes in the colicin gene clusters: colicin, immunity, lysis.

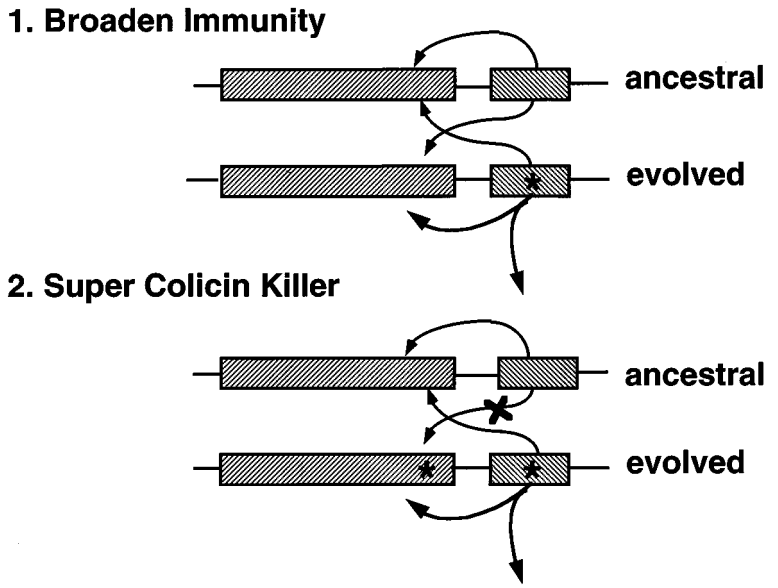

Figure 3 Illustration of the diversifying selection hypothesis. Two steps are hypothesized in the generation of novel colicin immunity function: *1.* generation of a broadened immunity in the evolved colicin gene cluster due to the starred point mutation in the immunity gene and *2.* generation of a super colicin killer due to the starred point mutation in the immunity binding region of the colicin gene. *Arrows* indicate the immunity relationships between the ancestral and evolved colicin gene clusters. An arrow from one immunity gene (*right hand box*) to one colicin gene (*left hand box*) indicates that the encoded immunity protein will protect against the encoded colicin protein.

immunity gene of a colicin that confers a broadened immunity function. Such a cell is immune to self, to its immediate ancestor, and to several other colicins (Figure 3). This evolved colicin gene cluster will have an obvious advantage over its ancestor under colicinogenic conditions and will be selectively retained in the population. While retained, a second, paired mutation in the evolved colicin gene results in a gene cluster that has a colicin to which its ancestor is no longer immune (Figure 3).

The strain carrying such an evolved colicin plasmid will have an immediate and large selective advantage. Positive selection will drive this "super killer" rapidly into the population, until some new colicin phenotype evolves from it in yet another round of diversification. Repeated rounds of this form of diversification of immunity function result in the accumulation of nonsynonymous substitutions in the immunity region as closely related colicins diverge.

Between rounds of immunity diversification, neutral substitutions will accumulate randomly along the colicin plasmid replicon. Recombination between the evolved and ancestral colicins will release neutral polymorphisms from their linkage with the selected sites and thus homogenize the evolved and ancestral colicin plasmid populations. However, recombination is lethal if it occurs between the pair of selected sites, as these sites together confer immunity specificity. Thus, a mutational trap is produced that results in the accumulation of neutral mutations in the immunity region (76, 80, 98, 99).

One line of evidence for the action of positive selection in nuclease colicin diversification comes from experimental studies. As described above, the diversifying selection hypothesis suggests that each round of diversification encompasses two steps: (*a*) the occurrence of coupled mutations in the immunity region that provide the host cell with an expanded immunity function and a novel colicin, and (*b*) the rapid fixation of this evolved strain in the ancestral colicinogenic population.

Studies on immunity specificity determinants of both RNase- and DNase-type colicins provide strong support for the feasibility of the first proposed step. Studies by Akutsu et al (2) on RNase colicins and by Curtis & James (16) on DNase colicins have narrowed the determinants for specificity of colicin:immunity interactions to very few residues. These data suggest that relatively few substitutions are required to generate novel immunity functions. In fact, this possibility was later confirmed by Masaki (63). He isolated a mutated colicin gene cluster that exhibited immunity to colicin E3 while maintaining its original immunity to E6. He further showed that such a broadened immunity function arose from a single point mutation, TGG (Trp-48) to TGT (Cys), in the immunity gene of colicin E6.

The second step in the diversification process, which requires the strain carrying the novel colicin gene cluster to rapidly invade the ancestral colicinogenic population, has also been examined experimentally (97). Several colicin-encoding plasmids naturally possess an additional immunity gene, which expands the hosts' immunity function in a fashion analogous to that described above. Invasion experiments reveal that when super killer strains (i.e. those with an additional immunity gene) are competed against their ancestors (i.e. those with only a single immunity gene), they rapidly displace the ancestral strain, even when initial frequencies of the invader are quite low (97).

The hypothesis of diversifying selection suggests that between events of diversification in immunity function, recombination with ancestral, or other closely related colicin plasmids, releases accumulated neutral substitutions from their linkage with the selected sites on the colicin plasmid. However, due to the specific interaction required between the immunity protein and the immunity binding domain of the colicin protein, recombination is successful only outside the immunity region.

Due to the low rates of recombination in *E. coli* this aspect of the diversifying selection hypothesis has not been directly addressed experimentally. However, results from a DNA sequence polymorphism survey provide some indirect evidence. A survey of colicin E2 polymorphism revealed two types of colicin E2 plasmids (98). One type was the classical E2 plasmid. The second type was a mosaic plasmid, apparently repeatedly derived by recombination between colicin E2 and E7. The recombinants had E2 immunity regions in otherwise E7 colicin plasmids. The existence of an intact E2 immunity region suggests that the specific interaction between the immunity protein and the immunity binding domain of a colicin selects against recombination events within the immunity region in order to maintain immunity function (98).

A further line of evidence for positive selection acting in colicin diversification comes from DNA sequence comparisons. Patterns of DNA polymorphism within species contain information about the evolutionary process that are not revealed from sequence divergence patterns (between species) alone. The combination of intra- and interspecific comparisons provides a powerful tool for investigating the evolutionary histories of DNA sequences (45, 56, 95). In particular, if sequences are evolving in a neutral fashion, the levels of inter- versus intraspecific divergence should be correlated. Regions evolving rapidly between species should accumulate polymorphisms rapidly within species (45, 64).

Patterns of DNA sequence polymorphism were examined for 14 colicin E2 gene clusters obtained from natural isolates of *E. coli* (98). These data were compared to the pattern of DNA sequence divergence between colicin E2 and its close relative, colicin E9. The patterns of polymorphism and divergence were not positively correlated. Statistical tests, including the HKA and MK tests (45, 64), which assess the correlation between the levels of polymorphism and divergence, were applied to these data and confirm a significant departure from neutral predictions.

Multiple unique recombination events across the immunity region could result in the observed patterns of substitution between colicins E2 and E9. However, during the time in which these multiple recombination events take place, neutral substitutions in the flanking regions should accumulate at an even faster rate, unless conjugation and recombination rates are many orders of magnitude higher in natural populations than has been suggested (37). Further, the availability of an appropriate recombination template is problematic given that the frequency of a specific colicin type is usually less than 1% in natural *E. coli* populations (1, 78). Finally, the specific interaction required between the immunity binding domain of the colicin and immunity protein would prevent recombination events within the immunity region that break up proper protein interactions. Thus, although the diversifying recombination hypothesis cannot be rejected, features of colicin biology make it a less likely explanation.

Recombination Drives the Diversification of Pore-Former Colicins

Pore-forming colicins differ significantly from nuclease colicins with respect to their killing action, translocation mechanisms, and, most relevant here, the action of their immunity proteins. Instead of directly binding to colicins in the cytoplasm as immunity proteins of nuclease colicins typically do, immunity proteins of pore-forming colicins are integral inner-membrane proteins (31, 50, 60, 62, 66, 67, 73, 87). They confer their function by intramembrane helix-helix interaction with hydrophobic transmembrane helices of the corresponding colicin channel within the lipid bilayer (31). Thus, structural constraints such as hydrophobic profiles rather than specific amino acid residues may be more important for the function of pore-forming immunity proteins.

Protein sequence comparisons suggest three main subfamilies of pore-former colicins. Colicins A, B, N, 28b, and U form a group, with a major hydrophobic segment of 49 residues in the pore-forming domain, while colicins E1, K, 5, and 10 comprise a second group, with a major hydrophobic segment of 38 residues (31, 60, 62). Colicins Ia and Ib are just about equally divergent from the A and E1 groups (62).

The colicin E1 group immunity proteins share three hydrophobic domains, which may correspond to three transmembrane helices found in the E1 immunity protein. On the other hand, the colicin A group immunity proteins share four hydrophobic domains, which may correspond to four transmembrane helices found in colicin A immunity protein (31, 50, 60).

Within the pore formers, the level of protein sequence similarity is quite low, rarely exceeding 40%. This high level of substitution has not noticeably altered pore former immunity hydropathy plots (Y Tan, unpublished information). However, given such a high level of divergence, it is not possible for a few mutations to produce cross immunity among the characterized pore formers, as was observed for the nuclease colicins. Rather than diversifying through the process of positive selection acting on immunity function, it appears that pore formers have followed a quite different evolutionary trajectory.

The most remarkable finding in the evolution of the pore-forming colicins is that regions of colicin sequence, encoding rather precise functional domains, have recombined to give rise to new colicins. Such recombination events occur both between and within the three groups of pore-forming colicins. Colicin B is frequently cited as an example of the power of multiple recombination events in colicin diversification (82, 86). Colicin B is composed of at least three segments from three origins: the colicin E1-like upstream region, the D-like N-terminal and central region, and the A-like C-terminal region (Figure 4).

Further support for recombination giving rise to new colicins comes from the work of Pilsl & Braun on colicins E1, 10, 5, and K (69, 70). Their comparative

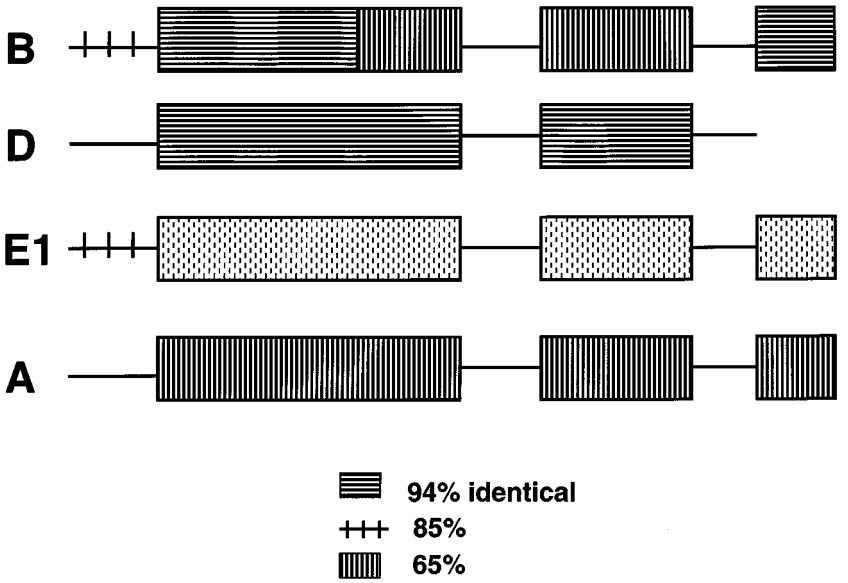

Figure 4 Levels of protein and DNA sequence similarity between colicin B and several other pore-former colicins. The different hatching patterns denote regions of protein and DNA sequence similiarity between B and the three other pore formers (D, E1, and A).

study of colicins E1 and 10 reveals a mosaic structure of colicin 10. E1 and 10 are similar in sequence in two domains that determine the common TolC requirement for uptake and pore-forming activity. E1 and 10 differ extensively in the remaining two domains that determine the Ton/Tol dependence and receptor binding.

Sequence comparison among colicins K, 5, and 10 reveals that these colicins were also assembled via recombination (69, 70). The N-terminal half of colicin K is derived from a source different from that of colicins 5 and 10. The C-terminal half of colicin 10 and the associated immunity and lysis proteins also have an origin from outside the E1 family of pore-former colicins.

Recombination also seems to have occurred between a chromosomally encoded bacteriocin and colicins. *Serratia marcescens* produces a chromosomally encoded bacteriocin, N28b, that shares a high degree of similarity in the first 45 residues with colicin A, with 35 identical residues (103). Moreover, alignment of the C-terminal amino acid sequence of 28b to the pore-forming domains of several colicins in the A family of pore formers reveals a high level of similarity distributed throughout the protein sequence (103). The hydrophobicity profile of the carboxy-terminal region of 28b shows a high degree of similarity to the corresponding domain of colicin A, and is characterized by a major

hydrophobic segment of 44 residues. Based upon levels of sequence similarity, 28b appears to be an evolutionary intermediate between the colicin A and E1 groups.

Recombination between functional domains may also explain the unusual pattern of divergence between colicins Ia and Ib. These are closely related colicins that show a high degree of substitution clustered in the immunity region, similar to the pattern seen for several nuclease colicins (76, 77). Studies of DNA sequence polymorphism for six colicin Ia plasmids reveal that patterns of divergence between colicins Ia and Ib and patterns of polymorphism within Ia were significantly different, with an elevated level of divergence in the immunity region and an even distribution of DNA polymorphism across the entire colicin gene cluster, again just like the nuclease colicins (80). This result contrasts with neutral predictions, which suggest a positive correlation between patterns of divergence and polymorphism (55). Unlike the case of nuclease colicin gene clusters, in which multiple recombination events are required to account for the observed patterns of clustered divergence, a single recombination event between Ia or Ib and some highly divergent colicin could produce the observed divergence pattern.

Why is recombination a dominant mode of pore former diversification? For diversifying recombination to occur, colicin plasmids must co-occur in the same host, be similar enough to permit recombination, and be divergent enough for recombination to result in a novel colicin phenotype. Pore-former colicins are by far the most abundant colicins. Approximately 80% of colicin producing strains produce pore-former colicins (1, 74, 78). Thus, co-occurrence of pore-former plasmids within the same host cell may be a frequent event, whereas it may be quite rare for the less abundant nuclease colicin plasmids. Note that in the one case in which nuclease colicins have been found at high frequencies in a population, recombination between two nuclease colicins is observed (98). Given the high level of protein sequence divergence between pore-former colicins, when recombination does occur it is likely to result in a novel colicin phenotype. Recombination among the closely related nuclease colicins is far more likely to result in almost indiscernible phenotypic effects. In fact, in the one case in which recombination between nuclease colicins has been observed, between colicins E2 and E7, the phenotype of the E2 chimera did not differ from the ancestral E2 phenotype (98).

Why is diversifying selection a dominant mode in the diversification of nuclease colicins? The first step in diversifying selection is hypothesized to require a mutation conferring broadened immunity. In the family of closely related nuclease colicins, broadened immunity can be achieved by one, or very few mutations in the immunity gene. In the highly divergent pore-former family of colicins, it is unlikely that broadened immunity can be easily generated.

Immunity proteins, and the regions of colicin proteins that interact with immunity proteins, are quite dissimilar in protein sequence among the pore-former colicins. Thus, the process of diversifying selection envisioned for nuclease colicins may simply not be possible within the pore-former family.

However, it is likely that pore-former colicins also experience some form of positive selection. One explanation for the high frequencies in which pore-former colicins are isolated from nature may be that as novel pore-former colicins are generated, they are quickly driven into *E. coli* populations through the action of frequency-dependent positive selection. Thus, pore former and nuclease colicins may not differ with respect to the importance of positive selection in their diversification, but rather in the molecular mechanisms involved in the generation of diversity. However, there are no data to directly assess the importance of positive selection on pore-former colicins. The recent finding of several relatively closely related pairs of pore-former colicins (colicin U, pCollet, 5, 10, and K) may allow this hypothesis to be directly addressed (69, 70, 89; J Neel, M Collet, M Adams, M Neely, DD Friedman, M Adams, L Cadavid & MA Riley, unpublished work).

Evolution of Colicin Resistance

Two primary mechanisms result in *E. coli* insensitive to the action of colicins, aside from the immunity mechanisms that the colicinogenic host encodes: (*a*) resistance involves the alteration of a cell surface receptor that binds a colicin and (*b*) tolerance involves alterations in cell membrane proteins involved in colicin translocation (18, 35, 61, 90, 112, 114).

Data from over 400 natural isolates of *E. coli* reveal that greater than 70% of the isolates are resistant to at least one colicin (33, 38, 78). A further 30% of the isolates are multiply resistant to all 18 colicins tested. Much higher levels of multiple resistance are observed than would be expected if one considers that only a small subset of colicins are currently segregating in the surveyed populations. In other words, within a population the identity of the segregating colicins and the colicin-resistance profiles do not match. In almost all cases, the resistant strains resist more colicins than are currently segregating. Are the observed high levels of multiple resistance due to the cumulative effects of past colicin selection events or could multiple resistances result from a single selection event?

All colicins enter a cell by recognizing and binding to a specific cell surface receptor. Loss of the receptor can confer resistance to all colicins that recognize that receptor. For example, mutations in the *btuB* locus, vitamin B12 receptor, can result in resistance to all nine E colicins (49, 72). However, other receptor mutations have been described that provide specific resistance to a single colicin or a subset of colicins (5). Mutations at loci involved in colicin translocation

can result in resistance to some or all colicins that share the same pathway (110). For example, mutations in the *ton*B locus can confer resistance to colicins B, D, G, Ia, Ib, M, and D (18).

A recent study was specifically designed to address the role of pleiotropy in the generation of colicin resistance; the production of multiple resistances from a single colicin exposure event (M Feldgarden & MA Riley, unpublished information). A total of 219 mutations were generated by exposure of a common *E. coli* K12 cell line to 1 of 11 different colicin extracts (A, D, E1–E8, and K). Ninety-six percent of the mutants were resistant to two or more colicins, with an average resistance to 11 colicins generated by a single colicin exposure. In no instance were mutants observed that were resistant to only one colicin. Translocation mutants (Tol pathway mutants) resisted more colicins than putative receptor (DOC^r) mutants. Thus, one explanation for the observed high levels of multiple colicin resistance is the occurrence of single-step multiresistant strains resulting from exposure to colicins (M Feldgarden & MA Riley, unpublished information).

It has often been assumed that colicin resistance imposes a fitness cost, by analogy to other types of resistance, including antibiotic and phage resistance (15, 58, 116). On average, there is a significant cost associated with colicin resistance; with an average reduction in maximal growth rate in rich media of 5% in a subsample of the colicin resistance mutants described above (M Feldgarden & MA Riley, unpublished information). This is comparable to the cost of antibiotic resistance (26), but three to five times less than the cost of phage resistance (58). There was considerable variation between resistant mutants with respect to growth rate effect. Mutants selected on the same colicin often had quite different growth rates, and there was no correlation between the number of colicins resisted and the cost of resistance. On average, translocation mutations were more costly, lowering maximal growth rates by 17%, whereas putative receptor mutations did not significantly lower growth rates (M Feldgarden & MA Riley, unpublished information).

There is one striking exception where the absence of pleiotropy might explain a colicin resistance pattern observed in natural populations. There is a significant lack of resistance to colicin E4 in natural populations. These lower levels of resistance are surprising since levels of resistance to other E colicins (E1–E9) that use the same receptor (*btu*B) and translocation pathway (*tol*QRA) are much higher. However, when resistance profiles were generated experimentally, colicin E4 resistance was the least commonly generated resistance among E colicins (M Feldgarden & MA Riley, unpublished information). Thus, the observed low frequencies of E4 resistance in nature may not result from colicin E4 rarity, but rather from the difficulty in generating resistance to that colicin.

Colicins are thought to play a role in intraspecific competition. If so, we are left with a paradox. If most *E. coli* resist the action of most colicins, how can colicin production be effective? It is possible that the high levels of resistance observed in the laboratory do not accurately reflect levels of resistance in nature, i.e. our assay for resistance is too sensitive. A recent study, employing natural isolates of colicin producers, found colicin titre differences between strains may explain the different patterns of resistance observed in the natural populations from which these strains were isolated (38). A regression analysis of the fraction of strains resistant to a particular colicinogenic strain against the titre of colicin produced by that colicinogenic strain revealed that 65% of the variation in resistance can be explained by colicin titre. This suggests that there may be situations in nature in which the levels of colicin produced are not as effective as we observe in the laboratory.

An alternative hypothesis argues that colicins may serve a role in interspecific, rather than intraspecific, competition. Colicins are narrow-range antimicrobials. However, they are effective against some isolates of the close relatives of *E. coli*. A preliminary investigation of this issue suggests that the levels of colicin resistance in other enteric bacteria may be even higher than levels observed for *E. coli* (M Feldgarden & MA Riley, unpublished information). However, few isolates from each of only six enteric species were examined. A more thorough, and phylogenetically based, investigation of this issue is warranted. What we require is a large sample of enteric species isolated from the same hosts at the same time. If co-occurring species have high levels of colicin resistance, the paradox remains.

BACTERIOCIN EVOLUTION BEYOND COLICINS

There is a rich literature reporting bacteriocin presence in bacteria spanning the entire spectrum of Eubacteria diversity. More recently, bacteriocin-like antimicrobials have been described for members of the Archaebacteria as well (50, 101, 104). However, there are surprisingly few studies that attempt to understand this diversity from a phylogenetic perspective, aside from those studies focused on the colicins of *E. coli*. In the following section we discuss one group of bacteriocins that have received some preliminary evolutionary attention, the pyocins of *P. aeruginosa.* Finally, we discuss one group of bacteriocins that, although only recently discovered, hold great promise in our quest to understand the evolutionary origins of bacteriocins, the halocins of Halobacteria.

The Pyocins

An extensive literature exists characterizing the bacteriocins of *Pseudomonas aeruginosa* (53, 57). Pyocins have been classified into three types based on their

structures: types R, F, and S. It has also been suggested that the heterogeneous R and F pyocins are defective phage, as they have structures similar to those of certain bacteriophage tails as visualized under the electron microscope, are easily sedimented by ultracentrifugation, and are resistant to proteinase treatment (53). The more homogeneous S pyocins are a group of simple proteins, of low molecular weight, and sensitive to proteases, that are not sedimentable or resolved under the electron microscope. The proteinaceous characteristics of S pyocins closely resemble those of colicins (39, 40, 85).

Pyocin typing of more than 1400 *P. aeruginosa* isolates from environmental and clinical sources has indicated that more than 90% produce one or more pyocins (20, 115). R and F pyocins are produced by over 90% of clinical isolates. S pyocins are produced by over 70% of these same strains. In a survey of pyocinogenic strains isolated from hospital counters, mops, and air filters, 54% produced S pyocins (36).

There are four S pyocins for which the pyocin and immunity genes have been characterized and sequenced (23, 85). S pyocins share functional similarity, nuclease activity, and some DNA sequence similarity with a subclass of the nuclease colicins of *E. coli* (23, 85). However, S pyocins are chromosomally encoded, whereas nuclease colicins are always plasmid encoded. It is not clear why bacteriocin genes are carried on different types of replicons in different bacterial species. Contrasting the mode of evolution of the putatively phylogenetically related pyocins and nuclease colicins may shed some light on this issue.

Pyocin proteins are organized into functional domains, just as is observed for the nuclease colicins. However, the order of the receptor recognition and translocation domains is reversed relative to colicins. The N-terminal region of the protein is involved in receptor recognition, followed by translocation and then nuclease and immunity binding domains. Figure 5 presents pairwise comparisons of four S pyocin and immunity proteins (23, 83–85; T Pinou & MA Riley, unpublished information). High levels of protein sequence similarity are observed between S1 and S2 in both the killing domain of the pyocin and the immunity protein, while much of the receptor binding domain and some of the translocation domain of this pair of pyocins are unalignable. A reduced level of protein sequence similarity is observed in comparisons of S1 or S2 with AP41, although the pattern of similarity is the same, i.e. higher levels of similarity in the immunity protein and the C-terminal end of the pyocin protein. S3 is the most divergent of this group, with the majority of the pyocin or immunity protein unalignable with S1, S2, or AP41. However, S3 does have a stretch of 70 amino acids in the killing domain that are 70% similar to a corresponding stretch in S1 and S2. It is unclear at this point whether the unalignable regions observed among S pyocin proteins represent divergent stretches of DNA saturated by

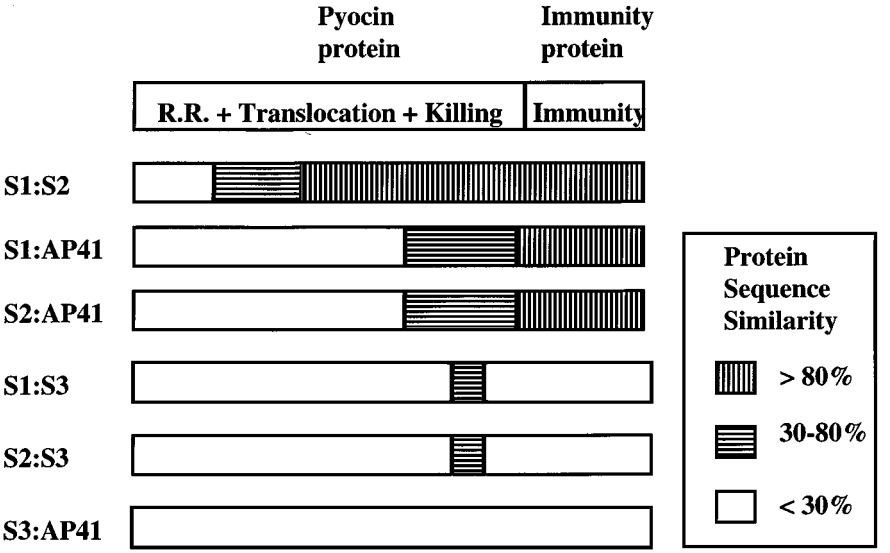

Figure 5 Levels of protein sequence similarity between pyocin and immunity proteins of *Pseudomonas aeruginosa*.

multiple substitutions but sharing a common ancestor, or tracts of recombined sequence from unrelated proteins.

The patterns of divergence among S pyocins are quite different from the patterns of divergence of their closest characterized relatives, the nuclease colicins. Substitutions between nuclease colicins are clustered within the immunity protein and the killing domain of colicin protein. Substitutions between pyocins are clustered within the translocation and receptor binding domains. In fact, the pattern of pyocin substitutions are more similar to patterns of substitution observed among the unrelated pore-former colicins. The pore formers are often highly divergent in the translocation and receptor binding domains, just as we observed among S pyocins.

We have previously made the distinction that nuclease and pore-former colicins experience very different evolutionary forces in their processes of diversification. The nuclease colicins appear to experience strong diversifying selection while the pore formers appear to experience diversifying recombination. It was argued that the distinction between these two modes of evolution may have less to do with the functional differences between nuclease and pore formers, but rather may simply reflect their relative frequencies in natural populations. The more abundant pore formers have an increased opportunity for recombination

than the relatively rare nuclease colicins. It was suggested that, as nuclease colicins increase in frequency in natural populations, recombination may become a more dominant mode in nuclease diversification (99). This may explain why S pyocins have such different patterns of evolution from their closest colicin relatives, the nuclease colicins. With pyocins at such high frequencies, diversifying recombination, rather than positive selection may be a more dominant force in pyocin evolution. To address this issue more directly we require intermediates in the pyocin phylogeny.

At present, the S pyocins are too divergent to allow robust sequence alignments and thus we have little power in reconstructing their evolutionary relationships or determining their modes of evolution. Given the high frequencies with which S pyocins are observed in nature it is surprising that additional phenotypic diversity has not been observed. This lack of diversity, relative to the high levels of diversity observed among the much rarer colicins, argues that we may not be able to generalize from the observed colicin modes of evolution.

Recently, a DNA sequence has been obtained for a bacteriocin isolated from *Klebsiella pneumoniae*, klebicin B (T Pinou, Y Wu & MA Riley, unpublished observations; 47). Klebicin B shows extensive sequence similarity to both pyocins and nuclease colicins. It appears to be most closely related to the nuclease colicins. However, as is the case for many pore-former colicins, the klebicin B gene cluster appears to be chimeric, with different portions of the gene cluster arising from pyocin-like and colicin-like sources. As more klebicin sequences become available, we may be in a better position to reconstruct pyocin, klebicin, and colicin phylogenetic relationships and to infer their modes of evolution.

The Halocins

The Halobacteriaceae are extremely halophilic Archaebacteria that inhabit hypersaline environments (65, 81). Recent studies have suggested that bacteriocin production is ubiquitous, perhaps even universal, within this group of bacteria (81, 88, 100, 101). Two halocins have been characterized in some detail, halocins H4, H6, and two putative microcins Hal R1 and S8.

Halocin H4 is apparently encoded on a megaplasmid and acts at the level of the membrane. It has a signal peptide that is cleaved from the preprotein upon secretion. This is quite different from the colicin-like proteins that do not have leader sequences and from the gram-positive proteins that have cleavable leader peptides that do not conform to typical signal sequences (46, 50). Halocin H6 shares several characteristics with H4: activity is maximal as the culture enters stationary phase, production is not induced by exposure to UV light, killing is by single-hit kinetics, and the protein is about 32 kDa (88).

A single halocin DNA sequence has been reported (S4) (13) and the protein sequence of one microcin sequence has just been deduced (S8) (R Shand,

personal communication). There is no detectable sequence similarity between S4 and any other characterized bacteriocins. For the moment, the small size of halocins and presence of a leader sequence ally halocins more closely to the bacteriocin of gram-positive bacteria. However, the leader sequences of gram-positive bacteriocins do not conform to typical signal sequences (46), as does the leader sequence of S4 (13). Attempts to discover if there are direct evolutionary ties between the halocins of Archaebacteria and one or more of the many different classes of bacteriocins of Eubacteria await the availability of additional halocin sequence information.

Regardless of whether halocins and other bacteriocins share a common ancestor, halocins apparently play a similar role in populations as that envisioned for traditional bacteriocins. Halocins are produced as the cells enter stationary phase. Thus, during a phase of limited resources, halocin-producing cells can lyse sensitive cells and enrich the nutrient content of the local environment (88). As stable proteins, they are hypothesized to remain in the environment long enough to then effectively reduce competition during subsequent phases of nutrient influx (88). It has been suggested that the stability of halocins may help explain why there is so little species diversity in hypersaline environments (65, 88).

CONCLUSIONS

Future Directions

The colicins of *E. coli* have served as a useful model for exploring the molecular mechanisms that generate high levels of bacteriocin diversity. Hypotheses that invoke either diversifying selection or recombination coupled with selection have been invoked to explain the patterns of nuclease and pore-former colicin diversity, respectively.

Several areas of investigation remain to develop colicins as a more complete model of bacteriocin evolution. A recently characterized pore-former plasmid, pCollet, was detected in an *E. coli* isolated from a Yanomama Indian (J Neel, M Collet, M Adams, M Neely, D Friedman, L Cadavid & MA Riley, unpublished information). DNA sequence determination of the entire colicin plasmid has revealed unexpected stability in the plasmid "backbone," compared to colicin plasmids E1 and A, even while the colicin gene cluster itself appears to be a chimera with multiple colicin ancestries. Investigation of colicin plasmid evolutionary history remains a virtually unexplored territory.

Perhaps most critical to our further understanding of colicin evolution is to determine the precise role colicins play in microbial interactions. Various studies provide conflicting data with respect to levels at which colicins act. Do colicins mediate population-level or species-level interactions? If colicins

mediate species-level dynamics, which species are involved? If we knew the players in this microbial arms race, we could explore in far greater detail the molecular mechanisms of coevolution between the toxins and the corresponding modes of resistance.

Bacteriocins comprise a heterogeneous group of toxins. It is unlikely that the role bacteriocins play, or their modes of evolution, will be the same in these diverse bacterial species. As we learn more about bacteriocin abundance and distributions, we require studies specifically designed to investigate phylogenetic relationships among these proteins. We currently have no hypotheses regarding the origin(s) of bacteriocins. Current work on the halocins of Archaebacteria and the bacteriocins of gram-positive Eubacteria should help us to unravel the evolutionary ties, or lack thereof, among this diverse and abundant family of antimicrobials.

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