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Chimeric Nature of Two Plasmids of *Hafnia alvei* Encoding the Bacteriocins Alveicins A and B

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The complete nucleotide sequences of two bacteriocin-encoding plasmids isolated from *Hafnia alvei* (pAlvA and pAlvB) were determined. Both plasmids resemble ColE1-type replicons and carry mobilization genes, as well as colicin-like bacteriocin operons. These bacteriocins appear to be chimeras consisting of translocation domains from Tol-dependent colicins, unique binding domains, and killing and immunity domains similar to those of the pore-forming colicin Ia. Just as is found for colicin Ia, these *H. alvei* bacteriocins (alveicins) lack lysis genes. The alveicins are unusually small at 408 and 358 amino acids for alveicin A and B, respectively, which would make alveicin B the smallest pore-forming bacteriocin yet discovered. The pattern of nucleotide substitution in the alveicins suggests that the dominant forces in the evolution of their killing domains and immunity genes are neutral mutation and random genetic drift rather than diversifying selection, which has been implicated in the evolution of other colicins. Five of six bacteriocinogenic isolates of *H. alvei* were found to carry plasmids identical to pAlvA. Comparisons of the levels of nucleotide divergence in five housekeeping genes to the levels of divergence in their respective plasmids led us to conclude that pAlvA is transferring laterally through the *H. alvei* population relatively rapidly.

Bacteriocins are protein toxins produced by bacteria that kill their closest relatives (28). The most extensively studied bacteriocins are the colicins, which are produced by *Escherichia coli*. 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 (apparently independent) 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 (9).

Colicins are classified according to the nature of either the translocation or killing domain. Translocation domains are divided into two groups based on the cellular machinery necessary for their entry, the group A, or Tol-dependent, and the group B, or Ton-dependent, transport systems (2, 10). Killing domains fall into three groups based on the mode of lethality (3). 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. The operons of all nuclease colicins and most pore formers contain a lysis gene. Some pore formers, such as colicins Ia, Ib, and B, do not have an identifiable lysis gene in the operon (13, 19, 36). These pore former colicins reside on high-molecular-weight plasmids, and it has been hypothesized that they possess genes involved in colicin release elsewhere on the plasmid (37).

The driving force behind colicin evolution appears to be positive selection for an altered killing spectrum. This can be achieved by altering either the translocation or binding domain, so that new target cells are recognized, or the killing domain, so that formerly immune cells are killed. Several studies have examined colicin evolution in an effort to deduce the relative roles of recombination, positive selection, and genetic drift as mechanisms for generating diversity in colicin-like bacteriocins (3, 23, 24, 34). Analysis of these mechanisms is often hampered by the lack of flanking sequence data for the plasmid carrying the colicin operon. Well over 25 plasmid-encoded colicin-like bacteriocins have been sequenced; however, in only eight cases has the entire plasmid sequence been determined.

In this study, we have determined the complete DNA sequences for two closely related *Hafnia alvei* bacteriocin plasmids, which allows us to examine how both intragenic and intergenic recombination have shaped the bacteriocin operons. These colicin-like bacteriocins appear to be chimeras consisting of translocation domains from Tol-dependent colicins, unique binding domains, and killing and immunity domains similar to those of colicin Ia. Just as is found for colicin Ia, these *H. alvei* bacteriocins (alveicins) lack lysis genes. The patterns of nucleotide substitution in the alveicins suggest that neutral mutation and random genetic drift rather than diversifying selection are the dominant forces in the evolution of the killing domains and immunity genes.

MATERIALS AND METHODS

Strains, plasmids and culture media. The bacteriocin-producing and -sensitive strains of *H. alvei* used in this study are from a collection of environmental enteric bacteria isolated from wild Australian mammals (5). Information about the strains, including the geographic origins and hosts, is given in Table 1. The *E. coli* strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd5* (r^- m^-) F′(*proAB*

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TABLE 1. Strains of *H. alvei* used in this study

Strain	Host organsim	Location[a]	Plasmid
MISC163	*Phascogale tapoatafa*	WA	NA[b]
MISC230	*Antechinus bellus*	NT	pAlvA
MISC231	*Dasyurus hallucatus*	NT	pAlvA
MISC259	*Dasyurus hallucatus*	NT	pAlvA
MISC261	*Dasyurus hallucatus*	NT	pAlvB
MISC690	*Homo sapiens*	WA	pAlvA
MISC692	*Homo sapiens*	WA	pAlvA

[a] WA, Western Australia (Perth); NT, Northern Territory (Kakadu Nature Preserve).
[b] NA, not applicable.

TABLE 2. Killing spectra of alveicins

Strain	Sensitivity[a]	
	Alveicin A	Alveicin B
MISC163	−	+
MISC164	−	+
MISC177	+	++
MISC198	+	++
MISC240	+	++
MISC310	++	−
MISC569	+	++
MISC590	++	−
MISC761	+	++
MISC763	+	++
MISC765	+	++

[a] −, no killing; +, weak killing; ++, strong killing.

lacI^q*Z*ΔM15)] (Promega) was used as a host strain for cloning bacteriocin gene clusters into the phagemid pBluescript II SK(+) (Stratagene). Cells were cultured in Luria broth (LB) (Sigma) or on LB agar plates with the addition of ampicillin (50 μg/ml) where appropriate.

Bacteriocin production assays. Two standard methods were used to verify bacteriocin production. The first is a lysate-based method (21), which was originally used to identify bacteriocinogenic strains in the culture collection (26). The second assay method (the chloroform vapor method [21]) was used for screening plasmid libraries for bacteriocin production.

Cloning strategy. Total DNA preparations from strains MISC259 and MISC690 were made using the method of Maloy (11) modified as follows. Instead of recovering the DNA via centrifugation, the precipitated DNA was spooled onto sterile Pasteur pipettes and washed by dipping the spooled DNA in 70% ethanol for 30 s. The DNA was then resuspended in 200 to 300 μl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight at 4°C. We have found that this method extracts both high- and low-molecular-weight plasmids, as well as genomic DNA.

The DNA was partially digested using *Sau*3AI (New England Biolabs) and cloned into the *Bam*HI site of pBluescript II SK(+) using standard protocols (12). Ligation reaction mixtures were desalted by extracting them three times with 1 volume of chloroform and were then electroporated into NM522. Transformants were selected by plating them onto LB agar containing ampicillin and were then screened for killing activity as described above.

Sequencing strategy. Clones that demonstrated killing activity were sequenced by primer walking starting at the SK and KS vector-priming sites located in the multiple cloning site of pBlueScript II SK(+). Sequence data from the primer walking was then used to design PCR primers to probe the other bacteriocin-producing *H. alvei* strains. A total of six bacteriocin plasmids were sequenced.

PCR mixtures (50 μl) were prepared with 1.25 U of *Taq* polymerase (Applied Biosystems), 1× *Taq* polymerase buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM (each) deoxynucleoside triphosphate, 0.2 μM (each) primer, and 2 μl of template DNA from a boiled cell suspension.

PCR products were purified using the QIAquick PCR purification kit (Qiagen). Plasmid to be used as a sequencing template was prepared using a Qiagen plasmid minikit. DNA sequencing was performed on the ABI 377 DNA sequencer. Fifty to 100 ng of purified PCR product, or 400 to 500 ng of plasmid template, was added to sequencing reaction mixtures, and sequencing was performed using Big Dye chemistry according to Applied Biosystems standard protocols.

Sequence analysis. The entire DNA sequences of pAlvA and pAlvB were queried against the National Center for Biotechnology Information nonredundant database using both the nucleotide-BLAST (blastn) and translated-BLAST (tblastx) programs.

For each gene tree, a protein sequence was inferred from the DNA sequences and a protein alignment was produced using the CLUSTAL W algorithm (35) in MegAlign version 4.05 (Dnastar, Inc.). The protein alignments were then converted back to DNA alignments for phylogenetic inference. Maximum-likelihood trees were inferred using PAUP version 4.0b8 (32). Optimized parameters for the heuristic algorithm used for building maximum-likelihood trees in PAUP were generated by the MODELTEST program version 3.06 (18). Statistical support of the branch points was tested by performing 1,000 maximum-likelihood bootstrap replications using PAUP version 4.0b8.

A sliding-window sequence comparison was done between the aligned sequences of pAlvA and pAlvB using the ARCADIA program (http://www.yale.edu/turner/people/jwertz). The comparison calculated the percent sequence identity, considering gaps as mismatches, across a sliding window of 40 bp with a step size of 1 base. The same software was used to examine the levels of synonymous and nonsynonymous substitutions per site in the bacteriocin and immunity genes of closely related pairs of bacteriocins. For this analysis, sites containing gaps were removed from the alignment. The step and window sizes were 1 and 40 bases, respectively.

Nucleotide sequence accession numbers. The GenBank accession numbers for the plasmid pAlvA and pAlvB sequences are AY271828 and AY271829, respectively.

RESULTS AND DISCUSSION

The bacteriocin-producing isolates used in this study were identified in a survey of enteric isolates from wild Australian mammals (26). Of the 42 *H. alvei* isolates screened for a killing phenotype against a total of 396 isolates from seven enteric species, 7 were originally designated as bacteriocinogenic. It was later determined that the killing phenotype of one of those strains was due to a temperate bacteriophage. Two different killing phenotypes were found in the six remaining bacteriocin-producing *Hafnia* strains. Following the standard naming convention for colicins, we have designated the colicin-like operons that encode these two phenotypes alveicins A and B. Likewise, the plasmids that encode the alveicins are named pAlvA and pAlvB, respectively.

Killing spectra of alveicins A and B. Table 2 lists those enteric isolates of the 396 tested that were killed by either alveicin A or B. Of the seven enteric species tested, only *H. alvei* isolates (11 of 40 tested) were sensitive to these alveicins. The killing spectra of alveicins A and B overlap to a large degree, with each toxin killing 9 of the 11 sensitive isolates. Mitomycin C induction resulted in increased toxin release from strains carrying either alveicin plasmid. The levels of toxin production of alveicin A-producing strains varied, with strains MISC230 and MISC259 consistently producing the most potent lysates. No difference in the killing spectrum of alveicin A was observed when it was cloned and expressed in *E. coli*. The relative sensitivities of isolates listed in Table 2 are valid only for the comparison of different strains to the same alveicin. No attempt was made to quantify the specific activities of the alveicins, so the killing strength is not relatable between groups.

Plasmid pAlvA and pAlvB sequences. Plasmid pAlvA is 5,113 bp and 46.18% G+C and was sequenced from five of the six *H. alvei* strains examined (Table 1). Plasmid pAlvB is 5,216 bp and 46.97% G+C and was isolated from strain MISC261.

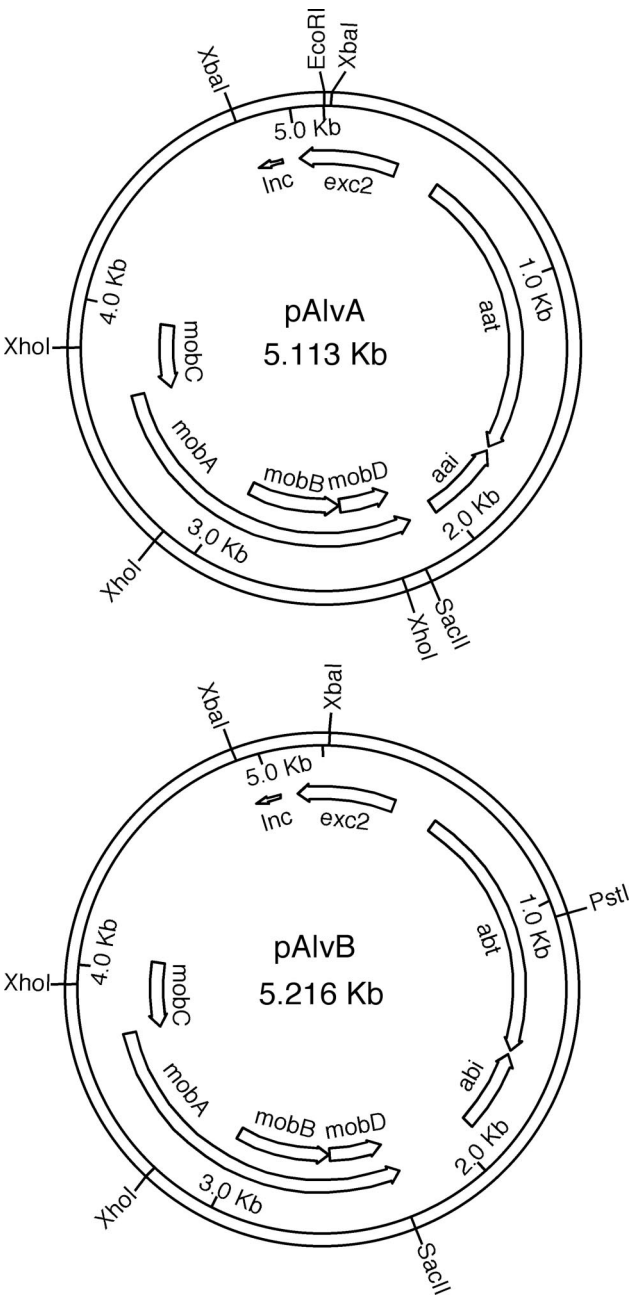

FIG. 1. Physical and genetic maps of pAlvA and pAlvB. The arrows indicate the positions and orientations of functional genes inferred from DNA and protein sequence comparisons. The arrows labeled Inc represent the predicted incompatibility region, with the direction of the arrow indicating the direction of RNA II transcription. Restriction sites for several common restriction endonucleases are indicated.

The pAlvA sequence is numbered starting at the unique *Eco*RI site in the presumptive *exc2* gene, which was identified by sequence similarity to the ColE1 gene of unknown function by the same name. To facilitate comparison, the pAlvB plasmid was numbered from the same location, even though the *Eco*RI site has been altered by a single base substitution. The two plasmids have very similar structures, as can be seen from their maps in Fig. 1. Through sequence comparisons with characterized plasmids, we have predicted the existence of seven protein-encoding genes in each of the alveicin plasmids. The location and relative orientation of each gene is given in Table 3. The overall nucleotide sequence similarity of the two plasmids is 86.5%. Differences between the two plasmids are not evenly distributed but are largely concentrated in two regions (Fig. 2). One region of elevated divergence falls within the toxin-encoding genes and strongly suggests that the binding domain and perhaps part of the translocation domain have been replaced in a recombination event in one of the plasmids. The second region of high divergence between the plasmids is the result of either the insertion in pAlvB or the deletion in pAlvA of 241 bases in the intergenic sequence between the bacteriocin operon and a cluster of mobilization genes.

Plasmid maintenance functions. Five of the seven predicted open reading frames (ORFs) encode plasmid maintenance functions. Table 3 lists the location, size, and both the protein and nucleotide sequence similarities for each of the encoded genes. Overall, the plasmid backbone resembles a colE1-type plasmid with putative promoters identified for RNAs I and II (Table 3). It was not possible to precisely predict the vegetative origin of replication because of the high degree of sequence divergence between these plasmids and known colE1-type plasmids in this region.

Plasmid organization. The relative orientations and positions of the alveicin operons in relation to the plasmid origins of replication are different from those found in other colE1-like colicin plasmids. All of the colicin and colicin-like plasmids that have been previously completely sequenced (colA, colD, colJs, cloDF13, colY, colE1, kleb B, and pesticin) have the same genetic organization with respect to the plasmid origin of replication. These colicin and colicin-like operons are positioned so that the colE1 origin of replication is immediately downstream of the colicin gene cluster, and the colicin toxin gene is transcribed in the same direction as the RNA II primer, which initiates plasmid replication. In the case of the alveicin plasmids, both the location and the orientation of the bacteriocin gene cluster differ from the norm. The *exc2* ORF is located between the origin of replication and the alveicin A and B operons, which are transcribed in the opposite direction from the RNA II primer (Fig. 1).

Analysis of bacteriocin operons. The regulatory regions of the alveicin A and B operons are typical of those found in most colicin-like plasmids. This similarity allowed us to infer the location of a σ^{70} promoter sequence, a LexA binding site, and a ribosome-binding site. The -35 and -10 sites of the promoter are located 106 and 83 bases upstream of the start codon, respectively. SOS control of the alveicins is likely mediated through the two overlapping LexA binding sites occupying the region 72 to 38 bases upstream of the first translated base. Sequence consistent with a ribosome-binding site is located 14 bases upstream of the start codon.

The alveicin operons both have the same organization. The immunity gene is located downstream of the toxin gene and is transcribed in the opposite direction, which is the case in all pore-forming colicins. Conspicuously absent is an ORF resembling a lysis gene. Colicin lysis genes, when present, are almost always located downstream of the immunity gene and are transcribed in the same orientation as the toxin gene. Lysis genes are quite small, ranging from 132 bp for colicins K, 5, and 10 to

TABLE 3. Nucleotide positions of inferred alveicin plasmid functional genes and their similarities to each other

Gene type	Gene	Location in[a]		Size (nuc[b]/aa)	pAlvA-pAlvB % similarity (nuc/aa)
		pAlvA	pAlvB		
Maintenance genes	*exc2*	5012 < 309	5115 < 309	411/136	89/89
	mobD	2220 < 2477	2312 < 2569	258/85	93/89
	mobB	2484 < 2948	2576 < 3040	465/154	95/93
	mobA	2182 < 3636	2274 < 3728	1,455/484	93/91
	mobC	3633 < 3956	3745 < 4048	324/107	92/93
	RNA I (−35)	4798 > 4803	4902 > 4907		
	RNA I (−10)	4822 > 4827	4926 > 4931		
	RNA II (−10)	4949 < 4954	5052 < 5057		
	RNA II (−35)	4972 < 4977	5075 < 5080		
Bacteriocin genes					
pAlvA	*aat* (Toxin)	491 > 1717		1,227/408	NA[c]
	aai (Immunity)	1734 < 2069		336/111	94/92
pAlvB	*abt* (Toxin)		490 > 1566	1,077/358	NA[c]
	abi (Immunity)		1583 < 1918	336/111	94/92

[a] The > or < sign indicates the relative orientation of the gene.
[b] Nuc, nucleotides.
[c] NA, recombination within the toxin genes makes overall similarity comparisons inappropriate for these genes. See the text for a detailed explanation of their relatedness.

198 bp for the colJs lysis gene (17, 30). There are 112 bp between the immunity gene and *mobA* in pAlvB and 355 bp between the same genes in pAlvA. A BLAST analysis of the 241-bp insertion in this region revealed sequence similarity to the *mob* region of pColD157 (6). In pColD157, the *mob* genes are situated on the 5′ end of the colicin operon, with the lysis gene found on the 3′ end of the gene cluster. It is therefore unlikely that either of these alveicins contains a lysis gene.

Alveicins evolved via recombination rather than diversifying selection. Recombination has been shown to be an important force in colicin evolution, both through domain swapping within the colicin gene and by moving the entire colicin operon to novel plasmid backgrounds. For example, in pColJs, the sequence flanking the colicin Js sequence resembles that of pPCP1, a pesticin-encoding plasmid from *Yersinia pestis*, while the remainder of the plasmid is 95% similar to pColE1 isolated from *E. coli* (30). Klebicin B, a colicin-like plasmid isolated from *Klebsiella pneumoniae*, appears to have undergone recombination both in the plasmid backbone and in the bacteriocin gene (27). The similarity of DNA sequences flanking the operon to sequences from pColA and pColE9, from *E. coli*, and from pyocin S1 on the chromosome of *Pseudomonas aeruginosa* suggests that the klebicin B operon is a chimera whose history involves movement from a colicin A-like plasmid into a *Klebsiella*-specific plasmid backbone and at some point acquiring a colE9/pyocin S1-like killing domain and immunity gene. Evidence of domain swapping is seen frequently in colicin genes; for example, colicins Ia and Ib share translocation and binding domains but contain different killing domains (34). Perhaps the most striking illustration of this form of functional diversification is the case of colicins 5, 10, and K, where the binding and translocation domains of colicin 5 are nearly identical to those of col10 whereas its killing domain is virtually identical to that of colK (17).

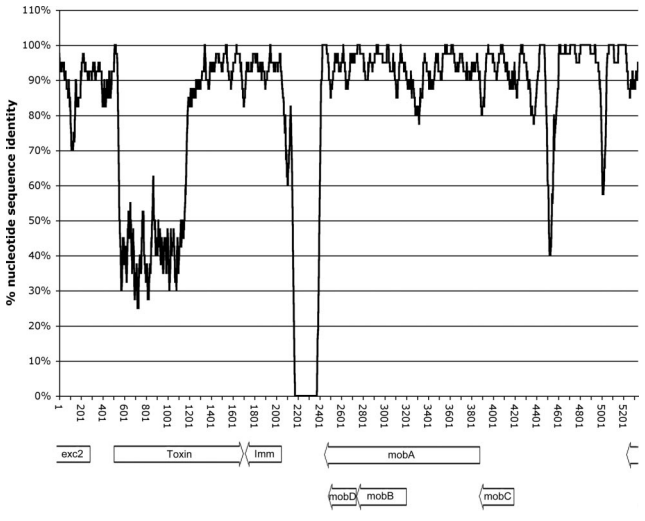

FIG. 2. Sliding-window nucleotide sequence comparison of pAlvA and pAlvB. The percent sequence identity was calculated in a sliding window of 40 bp with a step size of 1 base. Gaps in aligned sequences were treated as mismatches; there were no ambiguous bases. The arrows indicate the positions and orientations of inferred functional genes.

Alveicins A and B show evidence of several recombination events that have brought together two novel binding domains with translocation and killing domains similar to those seen in colicins. The amino-terminal translocation domains of alveicins A and B are similar to the Tol-dependent translocation domains of colicins E2 to E9 and cloacin DF13 (1, 4, 8, 14, 16, 31). A protein alignment of the translocation domains of these bacteriocins illustrates that the alveicins contain regions of sequence identity unique to both the E colicins and DF13 (Fig. 3). This pattern of sequence similarity suggests that either the alveicin genes have recombined in turn with DF13 and a member of the E group of colicins or they have exchanged sequence with an as-yet-unidentified recombinant of these two subgroups of bacteriocins.

The putative receptor binding domains of alveicins A and B are very different from each other. A BLAST analysis of these

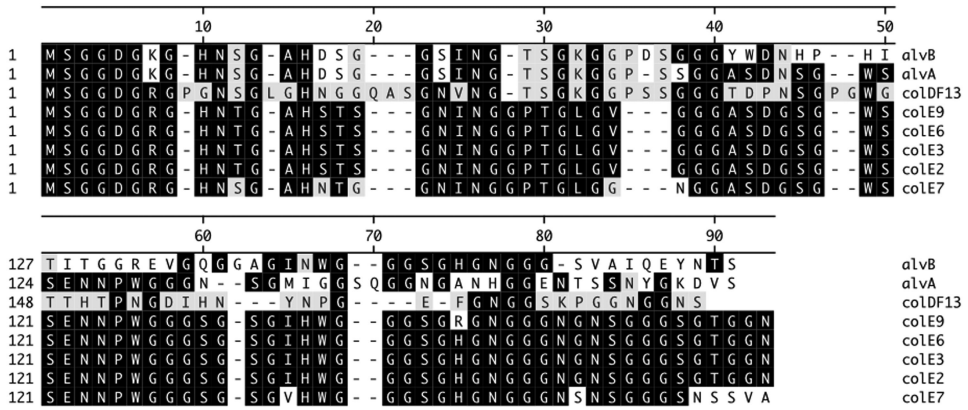

FIG. 3. Alignment of the amino-terminal regions of alveicins and Tol-dependent bacteriocins. Residues marked by solid boxes are identical to colE2, while those with shading are identical to cloDF13. The alveicins have stretches of sequence identity with both groups.

regions indicated no significant protein or nucleotide sequence similarity to any known sequence. This, coupled with the narrow killing spectrum of these bacteriocins, suggests that the bacteriocin attachment site may be an outer membrane protein specific to *H. alvei*. The binding domains are unusually short, making alveicin B, at 358 amino acids (aa), the smallest pore-forming bacteriocin yet discovered and alveicin A (408 aa) the third-smallest pore former, behind colN (389 aa) (20). Pore-forming colicins usually range from 490 aa for colB to 619 aa for colicins U and Y (25, 29).

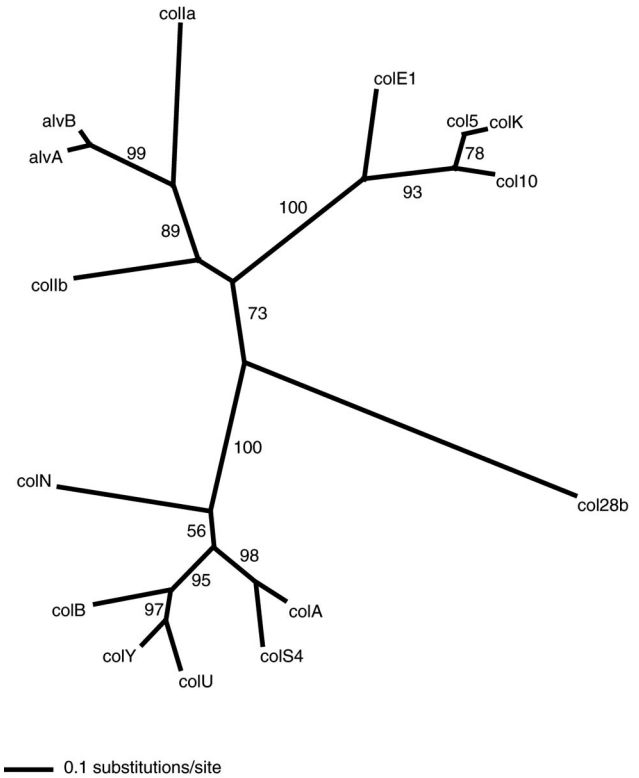

FIG. 4. Maximum-likelihood phylogram of pore-forming bacteriocin killing domains. Bootstrap values of >50% are indicated at appropriate branch points.

The carboxy-terminal killing domains and immunity genes of alveicins A and B are very similar to each other and are related to the colicin Ia and Ib killing domains and immunity genes (13). A phylogeny constructed using the killing domains of known pore formers demonstrates this relationship and further suggests that these four killing domains (those of colicins Ia and Ib and alveicins A and B) form their own monophyletic group (Fig. 4). None of the bacteriocin operons in this group are known to contain lysis genes.

Pairwise sequence comparisons of colicins that are believed to have experienced diversifying selection reveal a characteristic pattern of nucleotide substitution (34). Repeated rounds of positive selection will result in the accumulation of a disproportionately high ratio of nonsynonymous to synonymous nucleotide substitutions in the immunity gene and the carboxy-terminal killing domain of the colicin gene. The nucleotide substitution patterns seen in pairwise comparisons of colicins E3-E6, E2-E9, and U-Y best fit this model of colicin evolution (22, 25). This is demonstrated graphically in a sliding-window analysis of colicins E2 and E9 (Fig. 5A). Applying the same analysis to alveicins A and B demonstrates that the pattern of nucleotide substitution in the alveicin killing and immunity binding domains is inconsistent with the diversifying-selection model (Fig. 5B). The substitution pattern in the binding domains of the alveicins is what one would predict if recombination in this area had resulted in nonhomologous binding domains. Standard statistical tests for deviation from a neutral evolution model, such as the HKA (7) or MK (15) test, were not applicable because of the nature of our data. All five of the pAlvA plasmids are identical at the nucleotide level, and we have identified only one strain that carries pAlvB. Application of Tajima's test (33) to the nucleotide sequence alignment of the killing domains of all six genes was unable to reject the null hypothesis of neutral evolution ($D = -1.2497$, with $P \geq 0.1$). It should be mentioned, however, that Tajima's test depends on several assumptions that our data most likely violate, such as the equal mutability of all nucleotide sites.

We decided to investigate whether the homologous regions of the alveicin operons were diverging from each other at approximately the same rate as other genes carried on their plasmids. Table 4 contains the nucleotide diversity estimates

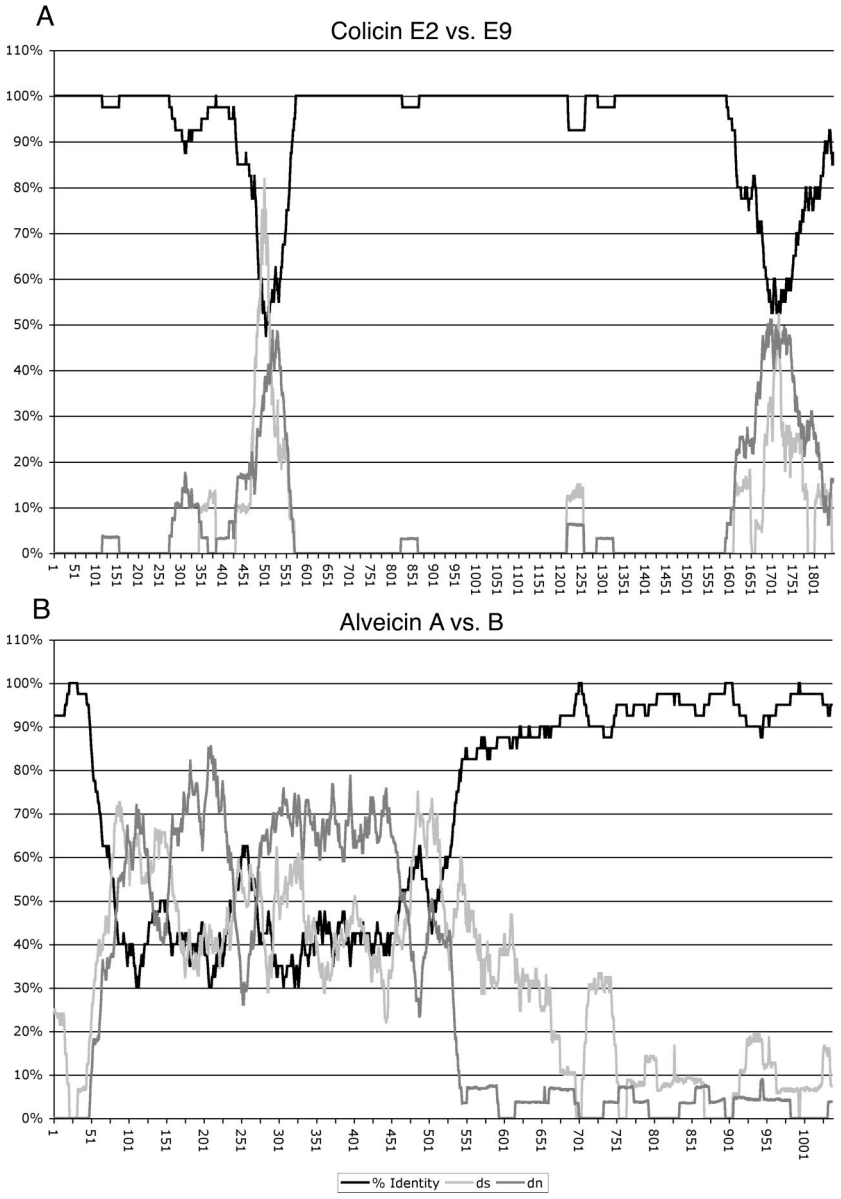

FIG. 5. Sliding-window pairwise comparison of bacteriocin genes. Comparisons were done between colicin E2 and E9 (A) and alveicins A and B (B). Calculations were performed on pairs of aligned sequences using a sliding window of 40 bp and a step size of 1 base. Gaps in aligned sequences were deleted from both sequences; there were no ambiguous bases. % Identity, percentage of nucleotide matches per sites compared; ds, percentage of synonymous substitutions per synonymous site; dn, percentage of nonsynonymous substitutions per nonsynonymous site. Values are not corrected for multiple substitutions per site. The *x* axis in all graphs is the number of the first base in the comparison window.

for all of the identified ORFs in pAlvA and pAlvB. Excluded from this comparison are the recombined binding domains of the bacteriocin genes, the 3′ 767 nucleotides of *mobA*, and the genes contained therein (*mobB* and *mobD*). The former were excluded because this type of analysis is meaningless for nonhomologous sequences, and the latter were excluded because we wished to avoid statistical artifacts introduced by the presence of multiple overlapping reading frames. Comparisons of levels of polymorphism between the alveicin killing domain and the plasmid maintenance genes using a χ^2 test indicate no significant differences in the levels of segregating polymorphisms among these genes ($0.2 < P \leq 1$). The same analysis was done for the immunity genes, and it indicated that their levels of polymorphism did not deviate significantly from those of plasmid maintenance genes ($0.05 < P < = 0.1$). These results, taken in conjunction with the results from Tajima's *D*

TABLE 4. Nucleotide diversity in alveicin plasmid-encoded genes

Gene	Length (bp)	No. of polymorphisms
Alveicin C-term	531	42
Imm	333	19
mobA N-term	685	53
mobC	321	23
exc2	408	45

test, indicate that either diversifying selection is not occurring in the alveicin killing and immunity binding domains or the level of selection is immeasurably small given the time of divergence for the two plasmids.

Evidence of rapid horizontal movement of alveicin plasmids. In the case of pAlvA, we found it striking that isolates collected from different hosts and geographic regions (collected ~1,600 miles apart) would contain absolutely identical plasmids (Table 1). This would imply that there is rapid intraspecies horizontal movement of the plasmid and/or that the plasmid is carried by an exceptionally fit strain of *H. alvei* that is able to invade and selectively sweep through local populations. To determine the extent to which each of these scenarios is most likely responsible for the high incidence of pAlvA-containing isolates, we examined the levels of nucleotide polymorphism in housekeeping genes in some of the isolates. As part of an earlier study, portions of six housekeeping genes were sequenced from the same collection of Australian enteric bacteria (38). Although *H. alvei* isolates were found to be generally more clonal than other enteric species, such as *E. coli*, pairwise sequence comparisons of *H. alvei* isolates carrying pAlvA indicate polymorphisms in all of the housekeeping genes examined with the exception of *gyrA*. The fact that we were unable to identify a single polymorphism in the five pAlvA plasmids we sequenced suggests that either the plasmid is evolving more slowly than chromosomal housekeeping genes or pAlvA is moving horizontally through the population at a rate which is rapid compared to the rate at which nucleotide substitutions accumulate. The latter seems more likely, especially given the amount of invariant noncoding sequence in pAlvA.

As more bacteriocins from diverse enteric species are characterized, more pieces of their evolutionary puzzle fall into place. The picture that is starting to emerge is one in which colicin-like bacteriocins are composed of functional modules drawn from natural plasmid libraries, where one of the functional modules is the plasmid backbone itself. If recombination between functional modules occurs via homologous recombination, as is suspected, then there would have to be a conserved universal interdomain sequence in which recombination occurs or there is a "linkage network" connecting subsets of modules that can recombine with each other. There is no evidence for the existence of a universal conserved sequence between domains. If such a linkage network exists, then the central receptor binding domain would govern which killing domain could be associated with a given translocation domain. Additional research in this area is necessary to determine the sizes and compositions of these module libraries, as well as the linkage relationships between specific families of modules, if any exist.

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REFERENCES

1. **Akutsu, A., H. Masaki, and T. Ohta.** 1989. Molecular structure and immunity specificity of colicin E6, an evolutionary intermediate between E-group colicins and cloacin DF13. J. Bacteriol. **171:**6430–6436.
2. **Braun, V., S. I. Patzer, and K. Hantke.** 2002. Ton-dependent colicins and microcins: modular design and evolution. Biochimie **84:**365–380.
3. **Braun, V., H. Pilsl, and P. Groβ.** 1994. Colicins: structures, modes of actions, transfer through membranes, and evolution. Arch. Microbiol. **161:**199–206.
4. **Cole, S. T., B. Saint-Joanis, and A. P. Pugsley.** 1985. Molecular characterisation of the colicin E2 operon and identification of its products. Mol. Gen. Genet. **198:**465–472.
5. **Gordon, D. M., and F. Fitzgibbon.** 1999. The distribution of enteric bacteria from Australian mammals: host and geographical effects. Microbiology **145:** 2663–2671.
6. **Hofinger, C., H. Karch, and H. Schmidt.** 1998. Structure and function of plasmid pColD157 of enterohemorrhagic *Escherichia coli* O157 and its distribution among strains from patients with diarrhea and hemolytic-uremic syndrome. J. Clin. Microbiol. **36:**24–29.
7. **Hudson, R., M. Kreitman, and M. Aguade.** 1987. A test of neutral molecular evolution based upon nucleotide data. Genetics **116:**153–159.
8. **James, R., M. Jarvis, and D. F. Barker.** 1987. Nucleotide sequence of the immunity and lysis region of the ColE9-J plasmid. J. Gen. Microbiol. **133:** 1553–1562.
9. **Konisky, J.** 1982. Colicins and other bacteriocins with established modes of action. Annu. Rev. Microbiol. **36:**125–144.
10. **Lazzaroni, J. C., J. F. Dubuisson, and A. Vianney.** 2002. The Tol proteins of *Escherichia coli* and their involvement in the translocation of group A colicins. Biochimie **84:**391–397.
11. **Maloy, S. R.** 1990. Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Inc., Boston, Mass.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Mankovich, J. A., C.-H. Hsu, and J. Konisky.** 1986. DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. J. Bacteriol. **168:**228–236.
14. **Masaki, H., and T. Ohta.** 1985. Colicin E3 and its immunity genes. J. Mol. Biol. **182:**217–227.
15. **McDonald, J., and M. Kreitman.** 1991. Adaptive protein evolution at the *adh* locus in *Drosophila*. Nature **351:**652–654.
16. **Nijkamp, H., R. deLang, A. Stuitje, P. vanden Elzen, E. Veltkamp, and A. van Putten.** 1986. The complete nucleotide sequence of the bacteriocinogenic plasmid CloDF13. Plasmid **16:**135–160.
17. **Pilsl, H., and V. Braun.** 1995. Strong function-related homology between the pore-forming colicins K and 5. J. Bacteriol. **177:**6973–6977.
18. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818.
19. **Pressler, U., V. Braun, B. Wittmann-Liebold, and R. Benz.** 1986. Structural and functional properties of colicin B. J. Biol. Chem. **261:**2654–2659.
20. **Pugsley, A. P.** 1987. Nucleotide sequencing of the structural gene for colicin N reveals homology between the catalytic, C-terminal domains of colicins A and N. Mol. Microbiol. **1:**317–325.
21. **Pugsley, A. P., and B. Oudega.** 1987. Methods for studying colicins and their plasmids, p. 105–161. *In* K. G. Hardy (ed.), Plasmids, a practical approach. IRL Press, Oxford, United Kingdom.
22. **Riley, M. A.** 1998. Molecular mechanisms of bacteriocin evolution. Annu. Rev. Genet. **32:**255–278.
23. **Riley, M. A.** 1993. Molecular mechanisms of colicin evolution. Mol. Biol. Evol. **10:**1380–1395.
24. **Riley, M. A.** 1993. Positive selection for colicin diversity in bacteria. Mol. Biol. Evol. **10:**1048–1059.
25. **Riley, M. A., L. Cadavid, M. Collett, M. Neely, M. Adams, C. Phillips, J. Neel, and D. Friedman.** 2000. The newly characterized colicin Y provides evidence of positive selection in pore-former colicin diversification. Microbiology **146:**1671–1677.
26. **Riley, M. A., C. M. Goldstone, and J. E. Wertz.** 2003. A phylogenetic approach to assessing the targets of microbial warfare. J. Evol. Biol. **16:**690–697.
27. **Riley, M. A., T. Pinou, J. E. Wertz, Y. Tan, and C. M. Valletta.** 2001. Molecular characterization of the klebicin B plasmid of *Klebsiella pneumoniae*. Plasmid **45:**209–221.
28. **Riley, M. A., and J. E. Wertz.** 2002. Bacteriocins: evolution, ecology and application. Annu. Rev. Microbiol. **56:**117–137.
29. **Schramm, E., J. Mende, V. Braun, and R. M. Kamp.** 1987. Nucleotide sequence of the colicin B activity gene *cba*: consensus pentapeptide among TonB-dependent colicins and receptors. J. Bacteriol. **169:**3350–3357.
30. **Smajs, D., and G. Weinstock.** 2001. Genetic organization of plasmid ColJs, encoding colicin Js activity, immunity, and release genes. J. Bacteriol. **183:** 3949–3957.
31. **Soong, B., F. Lu, and K. Chak.** 1992. Characterization of the *cae* gene of the ColE7 plasmid. Mol. Gen. Genet. **233:**177–183.

32. **Swofford, D.** 1997. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0. Sinauer Associates, Sunderland, Mass.
33. **Tajima, F.** 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics **123:**597–601.
34. **Tan, Y., and M. A. Riley.** 1997. Positive selection and recombination: major molecular mechanisms in colicin diversification. Trends Ecol. Evol. **12:**348–351.
35. **Thompson, J., D. Higgins, and T. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
36. **Thumm, G., T. Olschlager, and V. Braun.** 1988. Plasmid pColBM-CL139 does not encode a colicin lysis protein but contains sequences highly homologous to the d-protein (resolvase) and the oriV region of the miniF plasmid. Plasmid **20:**75–82.
37. **van der Wal, F. J., J. Luirink, and B. Oudega.** 1995. Bacteriocin release proteins: mode of action, structure, and biotechnological application. FEMS Microbiol. Rev. **17:**381–399.
38. **Wertz, J. E., C. M. Goldstone, D. M. Gordon, and M. A. Riley.** 2003. A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. J. Evol. Biol. **16:**1236–1248.