Plasmid **46,** 1–9 (2001)
doi:10.1006/plas.2001.1525, available online at http://www.academicpress.com on IDEAL

Nucleotide Polymorphism in Microcin V Plasmids

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Received December 21, 2000; revised March 26, 2001

DNA sequence polymorphism was determined for the microcin V gene cluster encoded on the microcin V plasmids of 12 natural isolates of *Escherichia coli*. These microcin V gene clusters are similar in DNA sequence, with only 10 of the 683 bp polymorphic. Further, the levels and patterns of microcin V gene cluster polymorphism differ from those of a chromosomal region, *trp*ORF2, sequenced from each of the host isolates. These contrasting levels and patterns of polymorphism suggest that the microcin V gene cluster has experienced an evolutionary history different from that of the host. © 2001 Academic Press

Key Words: bacteriocin; microcin; *E. coli*; polymorphism; microcin V.

Microcin V is a small toxic protein produced by enteric bacteria and encoded on a low-copy-number plasmid. It was first described as "factor-V" (Gratia, 1925), active in killing coliform bacteria, and then renamed "colicin V" (Fredericq, 1949). It has since become clear that colicin V is a microcin rather than a colicin. Unlike traditional colicins, microcin V is not SOS inducible, is of low molecular weight (less than 10 kDa versus 27–80 kDa for most colicins), and does not require a lysis protein for its release, but rather utilizes a set of dedicated export proteins (CvaA, CvaB, and Tol C) (Fath, 1992). Further, 15 amino acids are proteolytically digested from the N-terminus of the primary translation product upon maturation (Havarstein, 1994). The cleaved sequence of this posttranslational modification step is very similar to that of the small nonlantibiotic bacteriocins (class II) found in gram-positive bacteria such as *Pediococcus* and *Lactococcus* (Fremaux, 1995). The microcin V protein has a killing function similar to that of the pore-forming colicins (such as E1, Ia, and Ib) (Lazdunski, 1995).

The microcin V toxin is encoded by the microcin V gene (*cvaC*). The microcin V gene cluster comprises *cvaC* and a tightly linked microcin V immunity gene (*cvi*) that encodes a protein providing specific immunity to microcin V toxin. These two microcin-related genes overlap by 18 bp (Gilson *et al.*, 1990).

The microcin V plasmid belongs to the IncF1 incompatibility group (Waters, 1989; Waters and Crosa, 1991). Unlike other microcin and colicin plasmids, microcin V plasmids are found in high frequency among virulent enteric bacteria. Clinical surveys of septicemic and bacteremic *Escherichia coli* strains indicate that as many as 90% of these symptomatic strains harbor the microcin V plasmid (Waters and Crosa, 1991; Fernandez-Beros *et al.*, 1990; Cherifi *et al.*, 1994; Emery, 1992; Peighambari, 1995). The increased virulence of these strains has been explained by the presence of a gene encoding aerobactin on the microcin V plasmid (Fernandez-Beros *et al.*, 1990; Waters and Crosa, 1991), which results in increased iron utilization by the bacteria (Waters, 1986).

We report the entire nucleotide sequence of the *cvaC* and *cvi* genes for 12 microcin V plasmids isolated from two independent *E. coli* collections and one control strain (PAP1408) (Pugsley and Oudega, 1987). The levels of DNA sequence variability for microcin V *cvaC* and *cvi* genes are then compared to those reported for several colicin gene clusters (colicin E1 and Ia, Riley *et al.*, 1994; colicin E2, Tan and Riley, 1997; colicin Ib, Ayala *et al.*, 1994). In addition, levels of microcin V polymorphism are compared to those determined for a chromosomal locus (*trp*ORF2) from the same *E. coli* isolates.

0147-619X/01 $35.00

METHODS

Strains. This study sampled two unrelated collections of *E. coli*. The Achtman collection comprises *E. coli* isolates from European hospital patients collected over approximately 5 years (Achtman *et al.*, 1983). The five Achtman isolates included in this study represent five different serotypes, were isolated from three different countries (H42, H43, H68, Finland; H75, Holland; H83, England), and were collected over several years (H83, 1974; H42, 1975; H68, 1977; H43 and H75, 1979). The Whittam isolates were drawn from healthy chickens at a Pennsylvania farm and represent multiple isolates from three different chickens (chicken 910, C13, C23, C28; chicken 976, C54, C64, C94; chicken 1054, C73) collected over 4 months in 1987 (T. S. Whittam, personal communication). We have also included the microcin V-producing strain (PAP 1408) from the Pugsley collection, whose *E. coli* host is a derivative of K12 (Pugsley and Oudega, 1987).

DNA preparation and sequencing. Genomic and plasmid DNA were prepared according to standard methods (Ausubel *et al.*, 1992). The 526-bp sequence comprising the *cvaC* and *cvi* genes, along with 140 bases of 5′ and 17 bases of 3′ flanking sequence, was amplified. Primer sequences are available upon request. PCR conditions were 94°C for 1 min 20 s—1 cycle; 94°C for 30 s, 50°C for 40 s, 72°C for 30 s—30 cycles; 72°C for 1 min 20 s—1 cycle. The accession numbers for these plasmid sequences are AFO62844–846 for the Crosa strains and AFO62847–858 for all other strains. In addition, 293 bp of the tryptophan ORF2 (*yciF*) gene were amplified from the chromosomal DNA of each strain. Primer sequences are available upon request. PCR conditions were 94°C for 1 min 20 s—1 cycle; 94°C for 30 s, 70°C for 30 s, 72°C for 50 s—25 cycles; 72°C for 1 min 20 s—1 cycle. The accession numbers for these chromosomal sequences are AFO62832–843. All PCR products were separated on an agarose gel and the DNA was purified using GeneClean (BIO 101, Inc). Approximately 70–100 ng of this DNA was used for cycle sequencing according to the protocol of the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer).

Computer analysis. DNA sequence data were assembled and edited with Lasergene programs (DNASTAR, 1999). Phylogenetic trees were inferred using both neighbor-joining (Saitou and Nei, 1987) and parsimony algorithms PAUP 4.0 (Swofford, 1997). Confidence in the inferred parsimony tree topologies was assessed by application of a bootstrap algorithm based on 500 replications (Felsenstein, 1985). Levels of polymorphism were calculated using the MEA program (Moriyama, 2000). A goodness-of-fit (G) test was used to determine whether the levels of polymorphism were statistically different for the different genes and bacteriocins examined.

RESULTS AND DISCUSSION

Microcin V Nucleotide Polymorphism

DNA sequences of the 526-bp microcin V gene cluster (*cvaC* and *cvi* genes) and 157 bp of 5′ and 3′ flanking regions were determined for 12 isolates of microcin V-producing *E. coli* and one K12-derived microcin V-producing laboratory strain (PAP 1408) (Pugsley and Oudega, 1987). These DNA sequences were compared to the previously reported sequence obtained from a microcin V-producing laboratory strain (K12) (Gilson *et al.*, 1990).

There are 10 polymorphic sites in the 683-bp region examined (Fig. 1). Two of these polymorphisms are at synonymous sites in the microcin gene. One codon in the immunity gene has been multiply substituted, resulting in an amino acid replacement (Ala to Ile). There are six polymorphic positions in the 5′ region. The levels of polymorphism and nucleotide diversity estimated for this region are provided in Table 1. Excluding the short 3′ flanking region, levels of polymorphism range from a low of 0.65 and 0.85% in the *cvaC* and *cvi* genes, respectively, to a high of 4.29% in the 5′ flanking region.

The DNA sequences of the *cvaC* and *cvi* genes isolated from the five Europeans (H42, 43, 68, 75, 83) are identical to the PAP 1408 DNA sequence and the previously published

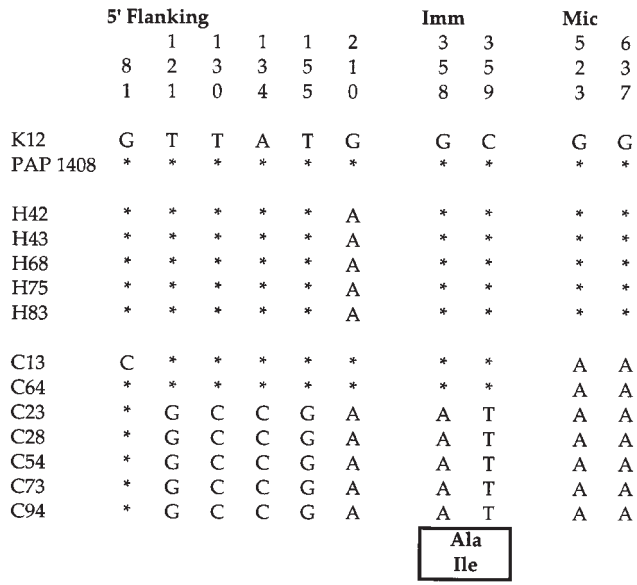

FIG. 1. Microcin V gene cluster polymorphism. Nucleotide positions are given above the polymorphic sites. Bold letters above numbers indicate each coding region. The box denotes the single amino acid substitution.

K12 DNA sequence (Gilson *et al.*, 1990). However, the human isolates all differ from PAP 1408 and K12 by one site in the 5′ flanking region. The human sequences are also quite similar to those obtained from two of the chicken isolates (C13 and C64), save for two polymorphic sites in the microcin gene (shared by C13 and C64) and one in the 5′ flanking region unique to C13. Due to the high levels of sequence similarity detected among these seven natural isolates, we have labeled this cluster of microcin sequences Group 1. Estimates of Group 1 polymorphism and nucleotide diversity are given separately in Table 1. The total nucleotide diversity observed in Group 1 is similar to the level of diversity estimated for the entire sample ($G = 3.0$, $df = 1$, $P > 0.05$).

The five remaining chicken isolates (C23, 28, 54, 73, 94) comprise the Group 2 cluster. These gene clusters are identical to each other and differ from Group 1 by two sites in a single codon in the immunity gene and four sites in the 5′ flanking region. Estimates of Group 2 polymorphism and nucleotide diversity are given separately in Table 1. The total nucleotide diversity observed in this group is significantly lower than the diversity estimated for the entire sample ($G = 14$, $df = 1$, $P < 0.05$).

Gene trees were inferred for the microcin V gene clusters. Distance-based and parsimony-based methods yield a single tree each with identical tree topologies and support the division of these microcin V gene clusters into Groups 1 and 2 (Fig. 2). Most of the polymorphism segregating for the microcin V gene cluster occurs between, rather than within, the groups.

trpORF2 Nucleotide Polymorphism

The *trp*ORF2 region on the host chromosome was chosen for DNA sequence comparison with the plasmid-encoded microcin gene cluster. This region appears to have evolved under the primary influence of random genetic drift and thus provides an estimate of the level of neutral polymorphism segregating on the host chromosome. DNA sequences of a portion of *trp*ORF2 was obtained for each of the strains. These sequences were compared to the previously published *trp*ORF2 sequence, positions 7838–8130 (Stoltzfus, 1988). There are 13 polymorphic

TABLE 1
Nucleotide Polymorphism and Diversity for the Microcin V Gene Cluster

Region	Total sites	Total poly	Total % poly	K_t	Syn sites	Syn poly	Syn % poly	K_s	Nonsyn sites	Nonsyn poly	Nonsyn % poly	K_n
Microcin V												
Total	700	10	1.43	0.006 ± 0.003								
3′	17	0	0	0								
Mic	309	2	0.65	0.003 ± 0.002	78	2	2.56	0.014 ± 0.008	231	0	0	0
Imm	234	2	0.85	0.004 ± 0.003	46	0	0	0	188	2	1.06	0.005 ± 0.003
5′	140	6	4.29	0.018 ± 0.009								
Group 1												
Total	700	4	0.57	0.002 ± 0.002								
3′	17	0	0	0								
Mic	309	2	0.65	0.003 ± 0.002	77	2	2.56	0.012 ± 0.009	231	0	0	0
Imm	234	0	0	0	46	0	0	0	188	0	0	0
5′	140	2	1.43	0.006 ± 0.005								
Group 2												
Total	700	0	0	0								
3′	17	0	0	0								
Mic	309	0	0	0	78	0	0	0	231	0	0	0
Imm	234	0	0	0	46	0	0	0	188	0	0	0
5′	140	0	0	0								
Human												
Total	700	0	0	0								
38	17	0	0	0								
Mic	309	0	0	0	78	0	0	0	231	0	0	0
Imm	234	0	0	0	46	0	0	0	188	0	0	0
5′	140	0	0	0								
Chicken												
Total	700	8	1.14	0.005 ± 0.003								
3′	17	0	0	0								
Mic	309	0	0	0	78	0	0	0	231	0	0	0
Imm	234	2	0.85	0.004 ± 0.003	46	0	0	0	188	2	1.06	0.005 ± 0.004
5′	140	6	4.29	0.019 ± 0.012								

Note. Abbreviations used as follows: Poly, polymorphism; Syn, synonymous; Mic, microcin; Imm, immunity; K_t, total nucleotide diversity; K_s, synonymous nucleotide diversity; K_n, nonsynonymous nucleotide diversity.

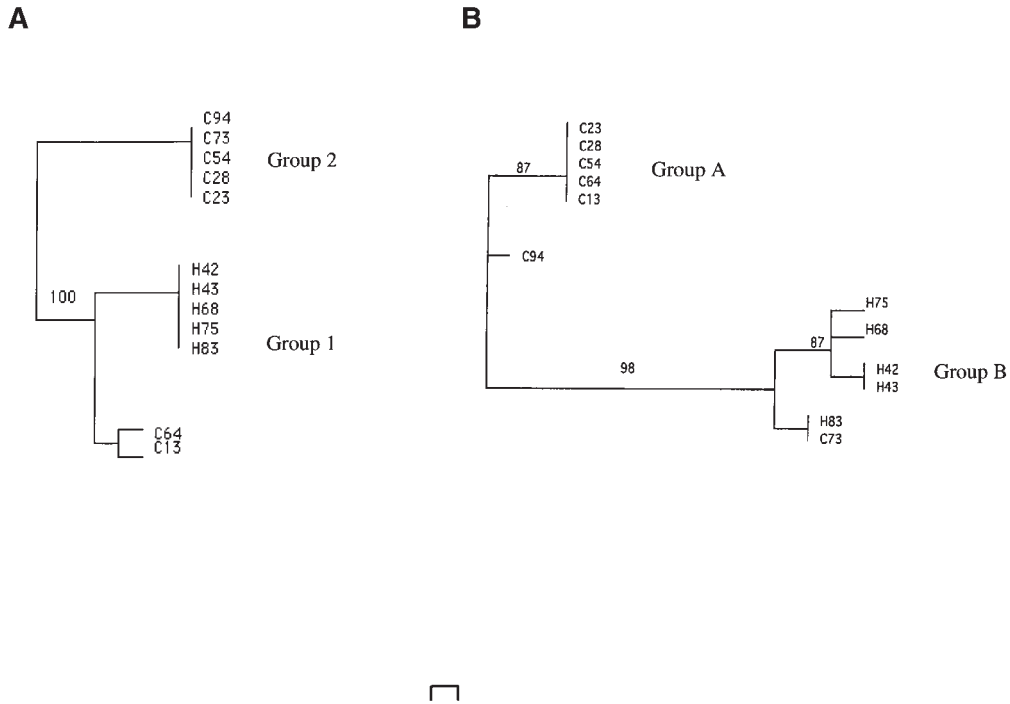

FIG. 2. Evolutionary trees inferred by parsimony methods based on (A) the polymorphic nucleotides of the microcin V gene cluster and (B) the polymorphic nucleotides of the *trp*ORF2 chromosomal gene. Bootstrap values above 80 are indicated. The scale bar equals 1 informative site.

sites in the 293-bp *trp*ORF2 region examined (Fig. 3). The estimated total nucleotide diversity is 0.021 ± 0.008 (Table 2). This level of nucleotide diversity is similar to estimates previously reported for this region ($G = 2.0$, $df = 1$, $P > 0.10$) (Whittam, 1993). The *trp*ORF2 sequences of PAP 1408 and C94 are identical to the published sequence. Five of the chicken strains are identical (C13, C23, C28, C54, C64) and differ from the published sequence at two positions. Five of the polymorphisms are nonsynonymous, resulting in amino acid substitutions. One of the chicken strains (C73) is more similar in sequence to the human strains and differs by as many as nine sites from the remaining chicken strains. Two of the human strains are identical (H42, H43), with the remaining three human strains (H68, H75, H83) differing at five positions (Fig. 3).

Gene trees were inferred for the *trp*ORF2 region. Distance-based and parsimony-based methods yield a single tree each with identical tree topologies and suggest two clusters of *trp*ORF2 sequences that differ in their topology from the microcin V gene cluster-based groupings (Fig. 2). Group A comprises all but one of the chicken isolates. Group B comprises sequences from all of the human isolates and a

trpORF2	7 8 4 6	7 8 4 9	7 8 5 7	7 8 5 8	7 9 2 9	7 9 9 5	8 0 2 4	8 0 0 4	8 0 5 9	8 0 7 1	8 0 7 8	8 1 0 5	8 1 2 0
pColV-K30	G	C	A	C	G	T	T	C	G	A	G	C	A
PAP1408	*	*	*	*	*	*	*	*	*	*	*	*	*
C94	*	*	*	*	*	*	*	*	*	*	*	*	*
C13	*	*	G	*	*	*	*	*	A	*	*	*	*
C23	*	*	G	*	*	*	*	*	A	*	*	*	*
C28	*	*	G	*	*	*	*	*	A	*	*	*	*
C54	*	*	G	*	*	*	*	*	A	*	*	*	*
C64	*	*	G	*	*	*	*	*	A	*	*	*	*
C73	A	A	*	*	*	C	*	T	*	G	*	T	C
H42	A	A	*	T	A	*	G	T	*	G	*	T	C
H43	A	A	*	T	A	*	G	T	*	G	*	T	C
H68	A	A	*	T	A	*	*	T	*	G	*	T	C
H75	A	A	*	T	A	*	*	T	*	G	A	T	C
H83	A	A	*	*	*	C	*	T	*	G	*	T	C
			Asn Ser		Ser Asn		Leu Arg				Glu Lys		Ile Leu

FIG. 3. *trp*ORF2 polymorphisms. Nucleotide positions are given above the polymorphism. The boxes below the sequences denote amino acid substitutions

single chicken isolate (C73). The estimated nucleotide diversity is significantly lower for each of these groups compared to the total diversity (Table 2) (for Group A, $G = 3.8$, $df = 1$, $P \leq 0.05$; Group B, $G = 8.6$, $df = 1$, $P < 0.05$). As with the microcin V gene cluster, essentially all of the variation is segregating between *trp*ORF2 groups, rather than within groups.

A comparison of the levels of polymorphism for the microcin V gene cluster (plasmid encoded) versus *trp*ORF2 locus (chromosomal encoded) reveals that the microcin cluster segregates significantly lower levels of polymorphism than does the *trp* region in this sample of *E. coli* isolates (total, $G = 7.4$, $df = 1$, $P < 0.05$; synonymous, $G = 7.07$, $df = 1$, $P < 0.05$; nonsynonymous, $G = 4.8$, $df = 1$, $P < 0.05$). The microcin V and *trp*ORF2 phylogenies are not congruent, with at least three putative horizontal transfer events suggested. These transfer events were simulated (Maddison and Maddison, 1992) by forcing the microcin V tree topology to be congruent with the *trp*ORF2 tree topology. This resulted in a doubling of the steps required to generate the tree. The inference of at least three transfer events in such a small sample of isolates argues that plasmid transfer may be an important force in the evolution of microcin V plasmids. Alternatively, it may suggest that *trp*ORF2 sequences have been exchanged between hosts.

There is significant structure in this sample of sequences. The DNA sequences obtained from human isolates form a cluster (both microcin V and *trp*ORF2), as do the majority of the isolates from chickens. For the microcin V sequences, there are two chicken isolates (C64 and C13) that cluster with the sequences isolated from humans. For the *trp*ORF2 sequences there is one chicken isolate (C73) that clusters with the sequences isolated from humans. It is not surprising that the DNA sequences from the chicken isolates would form a cluster as *E. coli* from chickens housed in the same coop were sampled (T. Whittam, personal communication). In fact, the DNA sequences for both loci sampled from most of these chicken isolates are nearly identical. This suggests that either chickens harbor very low levels of *E. coli* diversity or that there has been a recent selective sweep that brought a single *E. coli* strain (carrying a single microcin V gene cluster) to high frequency.

It is more surprising that the DNA sequences from the human isolates cluster. These *E. coli* isolates were collected from different hospitalized individuals in three different countries, sampled over a 5-year period. The *trp*ORF2 regions of these human isolates have accumulated significantly higher levels of polymorphism than their corresponding microcin V gene clusters, suggesting that the microcin related sequences and/or plasmid-encoded sequences, rather than the host strains, are more similar than expected if the two regions had shared a common evolutionary history.

The observation of identity among microcin V gene clusters in humans caused us to sequence three additional plasmids known to contain the microcin V gene cluster (pColV-CA7V, pB188, and pColV70) (Waters and Crosa, 1991). The pColV-CA7V plasmid was first isolated in 1942 by Fredericq *et al.* (1949), but there is no information regarding its original host. The pColV-CA7V microcin V gene cluster was identical to five of the chicken plasmids (which were themselves identical), save for one nucleotide substitution in the 5′ flanking region (bp 184). Thus, after more than 50 years, only a single substitution is observed in a noncoding region of the microcin V gene cluster. The two other microcin V gene sequences (pB188 and pColV70) are identical to the human microcin V sequences. Both of these microcin V plasmids were isolated 20 years ago from blood samples of bacteremic animals; pB188 was isolated from a cow and pColV70 from a chicken (Smith, 1976). These data suggest that there are at least two microcin V haplotypes that have maintained DNA sequence identity over a 20- or 50-year period.

We can rule out frequent fixation of the host strain in explaining the microcin V sequence identity among human and chicken isolates. The *trp*ORF2 region sequenced from each of the human isolates reveals significantly higher levels of polymorphism than do the corresponding microcin V gene clusters. Further, the chromosomes

TABLE 2
Nucleotide Polymorphism and Diversity of the *trp*ORF2 Region

Sample	Total sites	Total poly	Total % poly	K_t	Syn sites	Syn poly	Syn % poly	K_s	Nonsyn sites	Nonsyn poly	Nonsyn % poly	K_n
Total	293	13	4.4	0.021 ± 0.008	68	8	11.7	0.062 ± 0.023	225	5	2.2	0.009 ± 0.006
Group A	293	2	0.7	0.002 ± 0.003	68	1	1.5	0.005 ±0.008	225	1	0.4	0.002 ± 0.002
Group B	293	5	1.7	0.008 ± 0.006	67	2	3	0.016 ± 0.013	226	3	1.3	0.006 ± 0.005
Human	293	5	1.7	0.008 ± 0.006	67	2	3	0.012 ± 0.014	226	3	1.3	0.006 ± 0.005
Chicken	293	9	3.1	0.010 ± 0.008	68	7	10.3	0.033 ± 0.030	225	2	0.9	0.003 ± 0.003

Note. Abbreviations used as follows: Poly, polymorphism; Syn, synonymous; Nonsyn, nonsynonymous; K_t, total nucleotide diversity; K_s, synonymous nucleotide diversity; K_n, nonsynonymous nucleotide diversity.

TABLE 3
Nucleotide Polymorphism and Divergence Estimates for Several Bacteriocin Gene Clusters

Region	Total sites	Total poly	Total % poly	K_t	Syn sites	Syn poly	Syn % poly	K_s	Nonsyn sites	Nonsyn poly	Nonsyn % poly	K_n
Colicin E2												
Col	363	11	3.03	0.014 ± 0.006	71	6	8.45	0.047 ± 0.018	291	5	1.72	0.007 ± 0.004
Imm	261	7	2.68	0.009 ± 0.005	52	5	9.62	0.032 ± 0.021	208	2	0.96	0.003 ± 0.003
Colicin E1												
Col	1566	200	12.77	0.060 ± 0.02	333	124	37.24	0.180 ± 0.08	1233	76	6.16	0.03 ± 0.01
Imm	339	21	6.19	0.030 ± 0.01	62	10	16.13	0.080 ± 0.01	277	11	3.97	0.02 ± 0.01
Colicin Ia												
Col	1881	33	1.75	0.006 ± 0.002	577	16	2.77	0.009 ± 0.004	1304	17	1.3	0.004 ± 0.002
Imm	336	6	1.75	0.008 ± 0.003	69	4	5.80	0.029 ± 0.012	267	2	0.75	0.003 ± 0.001
Colicin Ib												
Col	528	10	1.89	0.005 ± 0.004	114	3	2.60	0.009 ± 0.008	413	7	1.69	0.004 ± 0.004
Imm	168	5	2.97	0.008 ± 0.008	31	2	6.50	0.023 ± 0.022	136	3	2.20	0.005 ± 0.006
Microcin V												
Mic	309	2	0.65	0.003 ± 0.002	78	2	2.56	0.014 ± 0.008	231	0	0	0
Imm	234	2	0.85	0.004 ± 0.003	46	0	0	0	188	2	1.06	0.005 ± 0.003

Note. Abbreviations used as follows: Poly, polymorphism; Syn, synonymous; Nonsyn, nonsynonymous; Col, bacteriocin; Imm, immunity; K_t, total nucleotide diversity; K_s, synonymous nucleotide diversity; K_n, nonsynonymous nucleotide diversity.

of the chicken isolates are distinguished by significant variation detected from a multilocus enzyme electrophoresis study (T. Whittam, personal communication). Thus, the microcin V gene cluster, rather than the bacterial host, is putatively being transferred and fixed so rapidly that it escapes the accumulation of neutral mutations.

In addition to the microcin V gene clusters considered here, four other colicin gene clusters have previously been the focus of DNA sequence polymorphism surveys (colicin E1 and Ia, Riley *et al.*, 1994; colicin E2, Tan and Riley, 1997; colicin Ib, Ayala *et al.*, 1994). The colicin E1 gene cluster appears to be the outlier in this group with respect to levels of total segregating polymorphism and divergence (Table 3). When colicin E1 gene clusters are removed from the analysis, the levels of total nucleotide diversity for colicins E2, Ia, and Ib and microcin V are not significantly different. Colicin E1 also exhibits significantly higher levels of synonymous and nonsynonymous diversity ($G = 97.6$, $df = 4$, $P < 0.05$ for synonymous sites; $G = 62$, $df = 4$, $P < 0.05$ for nonsynonymous sites). The high levels of polymorphism detected for colicin E1 plasmids may result from a larger plasmid population size or a more ancient origin (Riley *et al.*, 1994). Colicin E1 is a small, multicopy plasmid detected at high frequencies in all populations sampled to date (Gordon and Riley, 1992; Achtman *et al.*, 1983). Thus, the population size of the E1 plasmid may be much larger than that of the large, single-copy colicins or microcin V plasmids found at similar frequencies in natural populations and the relatively rare, low-molecular-weight colicin E2 plasmids.

Microcin V has been associated with strain virulence (Waters, 1986). This facet of its biology may help to explain the high levels of sequence identity observed for this sample of genes. Microcin V may experience frequent periodic selection events as it is transferred from one host to another. During each selection event, a single microcin V plasmid is fixed in the population, which results in the loss of all polymorphisms formerly segregating for the plasmid. However, this selection will not affect the level of variation segregating in the host chromosome. The target of selection during the periodic sweep, however, is unclear. To date, the presence of aerobactin, which has been associated with strain virulence (Fernandez-Beros *et al.*, 1990; Waters and Crosa, 1991), is the most obvious candidate. However, the microcin V plasmid is large (80–150 kDa) and contains many additional open reading frames which may potentially serve as targets for selection.

ACKNOWLEDGMENTS

We thank T. Whittam for providing the chicken strains and for access to the unpublished MLEE data on the chicken strains. We thank J. and L. Crosa for providing several microcin V strains. This work was supported by an NIH first award, DEB-9458247, NIH NIGMS R01 GM5 8433-01-04, and a grant from the General Reinsurance Corporation to M.A.R.

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Communicated by G. Archer