

# Evolution of Microcin V and Colicin Ia Plasmids in *Escherichia coli*<sup>∇</sup>

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**Survey results and genotypic characterization of *Escherichia coli* strains demonstrate that the bacteriocins colicin Ia and microcin V coassociate in a strain more often than would be expected by chance. When these two bacteriocins co-occur, they are encoded on the same conjugative plasmid. Plasmids encoding colicin Ia and microcin V are nonrandomly distributed with respect to the genomic background of the host strain. Characterization of microcin V and colicin Ia nucleotide variation, together with the backbone of plasmids encoding these bacteriocins, indicates that the association has evolved on multiple occasions and involves the movement of the microcin V operon, together with the genes *iroNEDCB* and *iss*, onto a nonrandom subset of colicin Ia plasmids. The fitness advantage conferred on cells encoding both colicin Ia and microcin V has yet to be determined.**

Bacteriocins are defined as antimicrobial proteins with a bactericidal action (30). Unlike traditional antibiotics that have a broad killing spectrum, bacteriocins are only toxic to the bacterial species they are produced by, and occasionally to closely related species (30). There is abundant evidence demonstrating that bacteriocins are important mediators of intra- and interspecies interactions and consequently a significant factor in maintaining microbial biodiversity (5, 20). Bacteriocins are also the subject of increasing attention as potential replacements for traditional antibiotics and as natural food preservatives (8). In addition, bacteriocinogeny is considered to be a key trait of strains used in probiotic formulations employed to prevent the establishment of microbial intestinal pathogens (32).

For those bacteriocins released via cell lysis, there is a wealth of mathematical theory (6, 7, 22), as well as in vitro (3, 12, 20) and in vivo (21) experimental evidence, that demonstrates the potential importance of bacteriocins in mediating intraspecific interactions and the highly dynamic nature of these interactions. However, our current understanding of the dynamics of bacteriocin production has largely concerned situations where the bacteriocinogenic population produces a single type of bacteriocin. Recently developed mathematical models predict that, under some circumstances, most of the strains of a bacterial community will evolve to produce multiple bacteriocin types (5). To understand the potential benefit arising from the production of multiple bacteriocin types, consider a community initially consisting of a sensitive cell population and two populations of producing cells, each encoding a single bacteriocin. If one of the producing cells acquires, through recombination, the genes for the other bacteriocin type, then this multiple-bacteriocin producer can kill sensitive cells and those cells encoding only a single bacteriocin. It is predicted that this outcome will occur when the costs associated with the production of multiple bacteriocins by a cell are not too severe, and

gene exchange among different producing strains is sufficiently frequent.

In *Escherichia coli*, the production of multiple bacteriocins by a single strain is a common phenomenon (10, 11). Several bacteriocins have been found to co-occur in strains more often than would be expected by chance, including the bacteriocins colicin Ia and microcin V (10, 11). Colicin Ia and microcin V are both encoded on high-molecular-weight plasmids varying in size from 80 to 150 kbp (37). Colicin Ia is a 70-kDa protein whose production is induced by the SOS system under times of stress (24). The microcin V protein is much smaller (<10 kDa), and its production is induced not by the SOS system but under conditions of iron limitation (26, 37). For both bacteriocins, the toxin gene is tightly linked to a specific immunity gene whose product protects the cell from the bacteriocin it produces (2). Though the mechanisms involved in inducing bacteriocin production vary, once produced, the colicin Ia and microcin V proteins behave similarly. Colicin Ia is an atypical colicin as it does not encode a lysis protein but utilizes export proteins that shunt the toxin out of the producing cell without destroying it, like a microcin. Once exported, both colicin Ia and microcin V attach to the Cir protein receptor (a siderophore receptor) on the outer membrane of target cells (26, 37). The TonB pathway moves the bacteriocins into the cell. Following entry, if no immunity protein is present for that particular bacteriocin, both colicin Ia and microcin V destroy the cell by forming pores in the affected cell's cytoplasmic membrane (2).

The purpose of this study was to further investigate the colicin Ia and microcin V coassociation. To accomplish this goal, mating experiments were performed to determine if the coassociation of these the two bacteriocins was a result of their being encoded on a single plasmid. Genotypic and phenotypic approaches, as well as nucleotide sequencing, were also used to gain additional understanding of the coassociation of these two bacteriocins within a strain.

## MATERIALS AND METHODS

**Strain collections.** To have as diverse a range of colicin Ia and microcin V plasmids from *E. coli* as possible, strains from three collections were examined. The first of these was a collection of *E. coli* strains isolated from nonhuman vertebrates living in Australia (9). The second collection represented clinical and

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fecal isolates taken from humans living in Canberra and its surrounds (14). The final collection consisted of antibiotic resistance plasmids originating from clinical and fecal isolates of *E. coli* taken from patients in Adelaide, Canberra, Melbourne, Perth, and Sydney hospitals (34). This R plasmid collection was produced by mating wild-type antibiotic resistance strains with *E. coli* K-12 strain J53 and then confirming that the transconjugants harbored a single plasmid (34). All of the strains used were stored as glycerol cultures at  $-80^{\circ}\text{C}$ .

**Strain characterization.** All of the isolates from the three collections had been assayed for a mitomycin C-inducible colicin phenotype by the method described by Gordon et al. (13) and screened by a PCR-based method for the presence of 11 colicins and seven microcins (10). The majority of *E. coli* strains can be assigned to one of four genetics groups, designated A, B1, B2, and D (15, 25, 38), and all isolates had been assigned to a genetic group by the method described by Clermont et al. (4). In addition, all strains had been screened by a PCR-based technique for the presence of 29 genes thought to enhance the ability of *E. coli* to cause intestinal and extraintestinal disease as described by Gordon et al. (14). The donor strains from which the R plasmid collection (34) was derived were also screened for the presence of the 18 bacteriocins and 29 virulence genes.

**Overnight cultures.** A subsample of a freezer culture was dilution streaked onto a Luria plate (Difco) and incubated overnight at  $37^{\circ}\text{C}$ . From this plate, a single colony was used to inoculate a flask containing 10 ml of Luria broth. The culture was incubated overnight at  $37^{\circ}\text{C}$  and shaken at 200 rpm.

**PCRs.** Template DNA was prepared with DNazol (Invitrogen) from a 50- $\mu\text{l}$  aliquot of an overnight culture according to the manufacturer's protocol. Amplification was performed with a 25- $\mu\text{l}$  reaction mixture containing 1.5  $\mu\text{l}$  of template DNA, 0.25  $\mu\text{l}$  of Platinum *Taq* (1.25 U), 1.5  $\mu\text{l}$  each of both the forward and reverse primers (10 pmol  $\text{ml}^{-1}$ ), 3  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$ , and 5  $\mu\text{l}$  of  $5\times$  buffer [67 mM Tris-HCl, 16.6 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.45% Triton X-100, 0.2 mg gelatin  $\text{ml}^{-1}$ , 0.2 mM deoxynucleoside triphosphates], with PCR grade water used to attain the required volume. Amplification conditions were 1 cycle at  $95^{\circ}\text{C}$  for 12 min; 25 cycles of  $94^{\circ}\text{C}$  for 30 s, 55 or  $63^{\circ}\text{C}$  for 30 s, and  $68^{\circ}\text{C}$  for 3 min; and 1 cycle at  $72^{\circ}\text{C}$  for 10 min.

**DNA sequencing.** Template DNA for sequencing of the colicin Ia and microcin V operons was obtained by the PCR protocol described above. Sequencing reactions used the Big Dye protocol and 3100 Genetic Analyzer from Applied Biosystems. A 1,200-bp portion of the 1,881-bp colicin Ia toxin gene was sequenced with primers Ia-F (5'-GGAATTCTCTTGACATGCCATTTTCTCCTT-3'), Ia657-R (5'-TCCTTCTGCCGTTTTTCAGCCTGTTTCGATT-3'), Ia658-F (5'-CGTATATCTCCCCGGAGGCCAGGTCGTTA-3'), and Ia1210-R (5'-TAACTTATCCCATTCAGCAACTTCAGCAAT-3'). The sequenced region for microcin V included 60 bp of the 5' flanking region, the 236-bp immunity gene, and 253 bp of the 311-bp toxin gene. The primers used were those described in reference 19. A 419-bp portion of the 558-bp plasmid gene involved in conjugative pilus synthesis regulation, *finO*, was sequenced for transconjugant strains positive for either colicin Ia or microcin V with the primers described in reference 1.

Sequences were edited and aligned with the Sequencher program. Maximum-likelihood trees were created with PAUP version 4.0b10 (36). Model parameters used for building maximum-likelihood trees in PAUP were generated by the MODELTEST program version 3.06 (27) by using the default starting parameters.

**Obtaining transconjugants.** PCR screening of the R plasmid transconjugant collection, as well as the strains originally harboring these plasmids, provided data concerning the extent to which bacteriocins Ia and V were encoded on the same plasmid. To obtain additional data on the frequency with which colicin Ia and microcin V were encoded on the same plasmid, further matings were performed by a previously described method (28). The donor strains were wild-type strains from animal and human hosts that had screened positive for both bacteriocins. A 100- $\mu\text{l}$  aliquot of an overnight culture of the donor strain together with 100  $\mu\text{l}$  of an overnight culture of *E. coli* K-12 strain CSH50 ( $\Delta\text{lac-pro gyr}$ ) were added to a flask containing 10 ml of Luria broth. The mating cultures were left in a nonshaking incubator for 2 nights at  $37^{\circ}\text{C}$ . A 1-ml aliquot of the mating culture was spread onto a selective plate (TL plates: Luria broth containing 50 mg 2,3,5-triphenyltetrazolium chloride  $\text{liter}^{-1}$ , 10 g lactose  $\text{liter}^{-1}$ , 100  $\mu\text{g ml}^{-1}$  nalidixic acid, and 11 g agar  $\text{liter}^{-1}$ ) and incubated overnight at  $37^{\circ}\text{C}$ . Following incubation, the plates were overlaid with 3 ml of Luria broth soft agar (6 g agar  $\text{liter}^{-1}$ ) containing 200  $\mu\text{l}$  of a CSH50 overnight culture and 80  $\mu\text{l}$  of mitomycin C (final concentration, 0.1  $\mu\text{g ml}^{-1}$ ) and then incubated at  $37^{\circ}\text{C}$  for another night. Colonies surrounded by a zone of clearing were dilution streaked onto TL plates containing nalidixic acid and onto minimal glucose plates (33) to confirm the identity of the putative transconjugants. In addition, each putative transconjugant was tested for colicin production and screened by a PCR approach for colicin Ia and microcin V. The transconjugant was deemed to be harboring a single conjugative plasmid if only a single band greater than 30 kb could be

TABLE 1. Frequency of *E. coli* strains encoding colicin Ia, microcin V, or both bacteriocins, together with the predicted fraction of strains encoding both bacteriocins assuming that they randomly associate<sup>a</sup>

Source and group	No. of isolates	% Observed			% Predicted, colicin Ia and microcin V	$P > \chi^2$
		Colicin Ia	Microcin V	Colicin Ia and microcin V		
Human						
A	90	4.4	3.3	6.6	1.1	<0.001
B1	51	9.8	7.8	2.0	1.1	0.575
B2	350	3.7	2.3	7.1	1.0	<0.001
D	128	4.7	2.7	3.1	0.4	<0.001
Animal						
A	93	2.2	1.1	0	<0.1	0.834
B1	272	6.2	1.8	1.1	<0.1	<0.001
B2	213	17.8	0.1	2.3	<0.1	<0.001
D	112	6.2	0	0		

<sup>a</sup> Data presented are for strains isolated from humans and nonhuman vertebrates living in Australia with respect to the genetic group membership of the strains.

detected when native plasmid gels were prepared by the in-well lysis method (34).

**Plasmid characterization.** All transconjugant strains shown to harbor plasmids encoding either colicin Ia or microcin V were further characterized. In addition to *finO* nucleotide sequence variation, the plasmids were characterized to determine if they belonged to the IncFII incompatibility group and if their *traY* gene was characteristic of an R- or F-type plasmid (1). The transconjugant strains were also screened for a number of putative virulence genes known to be plasmid associated: *iroN*, *traT*, *iss*, and *iutA* (19), as well as *sitA* and *tsh* (31).

The transconjugants were phenotypically screened for resistance to seven antimicrobials, i.e., ampicillin (10  $\mu\text{g}$ ), chloramphenicol (30  $\mu\text{g}$ ), kanamycin (30  $\mu\text{g}$ ), streptomycin (300  $\mu\text{g}$ ), sulfisoxazole (250  $\mu\text{g}$ ), tetracycline (30  $\mu\text{g}$ ), and trimethoprim (5  $\mu\text{g}$ ), with BD BBL Sensi-Disk antimicrobial susceptibility test disks according to the manufacturer's protocol.

## RESULTS

**Frequency of colicin Ia and microcin V in *E. coli*.** For this study, 1,308 *E. coli* strains were PCR screened for colicin Ia and microcin V operons. We found colicin Ia in 10% of the strains and microcin V in 5% of the strains. We detected colicin Ia in 24% and microcin V in 20% of the 72 transconjugant strains harboring R plasmids.

Strains of *E. coli* can be assigned to one of four genetic groups designated A, B1, B2, and D, and strains of these four groups appear to occupy different ecological niches and vary in their propensity to cause disease (9, 16). The probability of detecting colicin Ia in a strain depended on the host group from which the strain was isolated and the strain's *E. coli* genetic group membership [nominal logistic regression: human/animal,  $\chi^2_{(1)} = 2.80$ ,  $P = 0.094$ ; *E. coli* group,  $\chi^2_{(3)} = 19.70$ ,  $P = 0.0002$ ; human/animal \* *E. coli* group,  $\chi^2_{(3)} = 15.81$ ,  $P = 0.0012$ ]. Overall, there was no difference in the frequency of Ia-positive strains isolated from humans compared to non-human vertebrates (Table 1). In isolates from humans, the frequencies of colicin Ia were similar across the *E. coli* groups. By contrast, in strains isolated from animals, the frequency of colicin Ia-producing strains was significantly greater in strains belonging to genetic group B2 compared to strains of the other *E. coli* groups.

The likelihood of detecting microcin V in a strain also de-

pendent on the type of host it was isolated from [nominal logistic regression: human/animal,  $\chi^2_{(1)} = 27.54$ ,  $P = 0.0000$ ; *E. coli* group,  $\chi^2_{(3)} = 7.95$ ,  $P = 0.0470$ ; human/animal \* *E. coli* group,  $\chi^2_{(3)} = 15.81$ ,  $P = 0.0012$ ]. *E. coli* strains isolated from humans were significantly more likely to be microcin V positive than were strains isolated from nonhuman hosts (Table 1). Group D strains from both humans and animals were significantly less likely to be microcin V positive.

Strains encoding colicin Ia or microcin V and strains encoding both bacteriocins are present in each for the four major lineages of *E. coli* (Table 1). However, the distribution of microcin V is not independent of the genomic background of the host. Among the genetic group B2 strains isolated from humans, microcin V is never detected in strains that are positive for *focG*, *hlyD*, *iha*, and *she*. All microcin V-positive strains are also *kpsMTII* positive. Strains negative for *focG*, *hlyF*, *iha*, and *she* and positive for *kpsMTII* represent only 27% of the 350 group B2 strains, yet the prevalence of microcin V in strains with these characteristics is 35% (nominal logistic regression:  $\chi^2_{(1)} = 97.67$ ,  $P = 0.000001$ ) (15; unpublished data). All microcin V-positive strains are also positive for *traT*, *iss*, and *iroN*. However, *traT*, *iss*, and *iroN* can be detected in B2 strains positive for *focG*, *hlyD*, *iha*, and *she* and negative for *kpsMTII*.

**Coassociation of colicin Ia and microcin V.** Colicin Ia and microcin V coassociated within a strain significantly more often than would be expected by chance, irrespective of whether the strains were isolated from human or animal hosts [likelihood ratio test  $\chi^2_{(1)} = 127.24$ ,  $P < 0.0001$ ]. A significant coassociation was also observed in the collection of R plasmid transconjugants [likelihood ratio test  $\chi^2_{(1)} = 19.23$ ,  $P < 0.0001$ ]. Among the 72 R plasmids, 6% encoded only microcin V, 10% encoded just colicin Ia, and 14% encoded both bacteriocins.

A further analysis was undertaken to determine if the degree to which colicin Ia and microcin V were coassociated depended on the strain's *E. coli* group membership. This analysis was restricted to isolates from humans, as there were too few microcin V-positive strains isolated from animals. For this analysis, colicin Ia was used as the predictor variable. As expected, a strain that was colicin Ia positive was significantly more likely to be microcin V positive [nominal logistic regression: *E. coli* group,  $\chi^2_{(3)} = 1.75$ ,  $P = 0.6252$ ; Ia<sup>+/-</sup>,  $\chi^2_{(1)} = 19.65$ ,  $P = 0.0000$ ; *E. coli* group \* Ia<sup>+/-</sup>,  $\chi^2_{(3)} = 9.16$ ,  $P = 0.0272$ ]. However, the significant interaction term indicates that the extent to which the two bacteriocins were coassociated differed with respect to the *E. coli* group. The coassociation of these two traits was not observed in group B1 strains (Table 1).

It is unlikely that the coassociation of these two bacteriocins is an artifact arising because the same strain was sampled from many different hosts. First, each strain sampled was from a different host individual and, as a first approximation, different hosts harbor different strains of *E. coli*. Second, both bacteriocins are encoded by strains belonging to each of the four genetic groups of *E. coli*. Third, the bacteriocin and virulence gene screening reveals 26 distinct profiles among the strains encoding both colicin Ia and microcin V.

**Colicin Ia and microcin V are found encoded on one plasmid.** In total, 36 transconjugants were obtained from strains where the donor was found to be positive for microcin V,

colicin Ia, or both bacteriocins (Table 2). In all cases, when a transconjugant was obtained, the bacteriocin profile of the resulting transconjugant matched that of the donor strain and only a single plasmid band could be detected. In eight cases, a colicin Ia-positive transconjugant was obtained from a colicin Ia-positive donor and five microcin V-positive transconjugants were obtained from microcin V-positive donors. There were 23 transconjugants obtained from donor strains that were positive for both bacteriocins; in every case, the resulting transconjugant harbored a single plasmid and encoded both bacteriocins. These results indicate that when both bacteriocins are present in a strain they are most likely to be encoded on a single plasmid.

**Characterizing the bacteriocin-encoding plasmids.** The plasmids encoding only microcin V were very homogeneous; all were members of the FII incompatibility group and possessed an F-type *traY* gene, and only three *finO* alleles were detected (Table 2). By contrast, the plasmids encoding only colicin Ia were diverse (Table 2). Of the seven Ia plasmids, just two were IncFII positive and had an F-type *traY* gene. Each colicin Ia plasmid possessed a unique *finO* allele (Table 2). The plasmids encoding both bacteriocins were also diverse (Table 2). Only one of the F-type plasmids was a member of the IncFII group, and the balance of the IncFII plasmids had an R-type or underdetermined *traY* gene. Among the 23 transconjugants encoding both bacteriocins, nine *finO* alleles were observed.

The transconjugants were also PCR screened for five other genes known to be plasmid encoded. All plasmids encoding microcin V were also *traT*, *iss*, and *iroN* positive, while *sitA* was detected in 86% of the microcin V-positive strains (Table 2). Plasmids that encoded only colicin Ia were negative for *iutA* and *iucC*, while 71% of the microcin V-positive plasmids possessed these traits (Table 2).

The antibiotic resistance profiles of the bacteriocin-encoding plasmids were very diverse, with few plasmids having identical resistance profiles (Table 2).

**Microcin V sequence variation.** Fifty microcin V-producing strains were sequenced for 527 bp of the microcin V operon. The sequenced region included 60 bp of the 5' flanking region, the entire immunity gene (236 bp), and 253 bp of the 311-bp microcin V gene. The immunity gene and the toxin gene overlap by 22 bases, which accounts for the discrepancy in the values. The average proportion of nucleotide differences among the 50 sequences was  $0.0005 \pm 0.0003$ .

Only five sequence types (STs) were observed, and excepting five of the strains, all of the strains were identical to the published data (27) for the microcin V operon (Table 3). Two of the strains (H244 and R087) had a 15-bp duplication in the 5' flanking region, and one strain (H781) had a 15-bp deletion in the 5' flanking region.

**Colicin Ia sequence variation.** The colicin Ia toxin gene is 1,881 bp in length. A 1,124-bp region (including 1,092 bp of the colicin Ia gene and 32 bp of the 5' flanking region) from 69 strains was sequenced. Among the 69 sequences, there were 61 polymorphic nucleotide sites giving rise to 36 STs and 18 amino acid replacements. The average proportion of nucleotide differences among the 69 sequences was  $0.0065 \pm 0.0013$ . The phylogenetic relationships among the colicin Ia sequence types are depicted in Fig. 1.

Colicin Ia sequence types from strains encoding both bacte-

TABLE 2. Phenotypic and genotypic characteristics of plasmids encoding the bacteriocins colicin Ia and microcin V

Strain	Colicin Ia	Microcin V	<i>finO</i> ST	<i>TraY</i> type	IncFII	<i>traT</i>	<i>iroN</i>	<i>iss</i>	<i>sitA</i>	<i>iutA</i>	Resistance profile <sup>a</sup>
AE008	+	-	10	-	-	-	-	-	-	-	TMP SXT AM
CE015	+	-	14	R	-	+	-	+	-	-	TMP SXT AM S
PE006	+	-	6	-	-	-	-	-	-	-	TE
SE016	+	-	7	-	-	-	-	-	-	-	AM TE C
SE017	+	-	20	-	-	-	-	-	-	-	AM TE C
AE013	+	-	4	F	+	+	-	-	-	-	TMP SXT S SPT
CE003	+	-	18	F	+	+	-	-	-	-	TMP AM TE
AE011	-	+	5	F	+	+	+	+	+	-	TMP SXT AM S TE
CE014	-	+	3	F	+	+	+	+	+	+	TMP STX
PE007	-	+	5	F	+	+	+	+	+	-	TMP STX S
SE010	-	+	5	F	+	+	+	+	+	-	TMP STX
H772	-	+	15	F	+	+	+	+	+	+	TE
AE016	+	+	8	F	-	+	+	+	-	-	TMP STX
CE004	+	+	8	-	-	+	+	+	+	+	TMP STX AM S TE K
CE010	+	+	12	-	-	+	+	+	+	+	TMP STX AM S TE K
PE001	+	+	8	F	-	+	+	+	+	+	TMP STX AM S
PE002	+	+	8	F	-	+	+	+	+	+	AM TE
PE004	+	+	5	F	-	+	+	+	+	-	TMP
PE009	+	+	19	F	-	+	+	+	+	+	TMP STX AM S TE
H053	+	+	8	F	-	+	+	+	+	+	TMP SXT AM S
H070	+	+	8	F	-	+	+	+	+	+	TMP A S TE
H073	+	+	8	F	-	+	+	+	+	+	TMP A S TE
H111	+	+	8	F	-	+	+	+	+	+	SXT AM
H112	+	+	8	F	-	+	+	+	+	+	AM TE
H127	+	+	11	F	-	+	+	+	+	+	AM S TE
H188	+	+	8	F	-	+	+	+	+	+	TMP SXT AM S TE K
H190	+	+	8	F	-	+	+	+	+	+	TMP AM S TE K
H461	+	+	8	F	-	+	+	+	+	+	AM S
H549	+	+	8	F	-	+	+	+	+	+	TMP SXT AM S
H098	+	+	13	R	+	+	+	+	-	-	S
H100	+	+	9	R	+	+	+	+	-	-	TMP STX AM
H314	+	+	1	-	+	+	+	+	-	-	AM S
AE009	+	+	17	R	+	+	+	+	+	+	TMP STX
AE010	+	+	17	R	+	+	+	+	+	+	TMP STX TE
SE018	+	+	5	F	+	+	+	+	+	-	TE C

<sup>a</sup> AM, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; SXT, sulfisoxazole; TE, tetracycline; TMP, trimethoprim.

riocins occurred on multiple branches of the tree and encompassed much of the breadth of the observed colicin Ia nucleotide variation. This outcome suggests that the co-occurrence of colicin Ia and microcin V could have evolved multiple times. However, few of the nodes have strong bootstrap support, so a different approach was used to investigate whether all of the strains encoding both bacteriocins could have evolved from a common ancestor. To accomplish this, all of the colicin Ia- and microcin V-positive strains were constrained to be monophyletic and a new maximum-likelihood tree was estimated. Comparing the likelihood scores of the constrained and unconstrained trees revealed that forcing strains that produce both bacteriocins to be monophyletic resulted in a significantly worse fit to the data [Difference - ln(L) = 43.238,  $P < 0.0039$ ].

Inspection of Fig. 1 suggests that sequence variation in this region of the colicin Ia gene may be different in strains that also produce microcin V. Four populations were defined, Ia STs isolated from humans, Ia STs isolated from animals, Ia STs isolated from humans where microcin V co-occurred, and Ia STs from animals where microcin V also co-occurred. For the analysis, each population consisted of a single representative of each ST observed in that population. Molecular analysis of

variance (AMOVA) confirmed that a significant fraction of the observed colicin Ia sequence variation was explained by the source of isolation and whether microcin V co-occurred with colicin Ia in the strain (AMOVA:  $\Phi_{PT} = 0.246$ ,  $P < 0.001$ ). Pairwise comparisons revealed that the source of isolation did not explain a substantial amount of the variation in the colicin Ia sequences, and this was particularly true for strains where microcin V was also present (Table 4). However, a substantial amount of the colicin Ia sequence variation depended on whether microcin V was also present in the strain (AMOVA:  $\Phi_{PT} = 0.256$ ,  $P < 0.001$ ).

The majority (83%) of the colicin Ia sequences from strains where microcin V was also present had a methionine rather than an asparagine at position 92 and an alanine rather than a glycine at position 109 (Table 5). Further, a leucine at position 295 was only present in strains that were also positive for microcin V (Table 5).

## DISCUSSION

The microcin V operon clearly occurs with the colicin Ia operon more frequently than would be expected by chance.



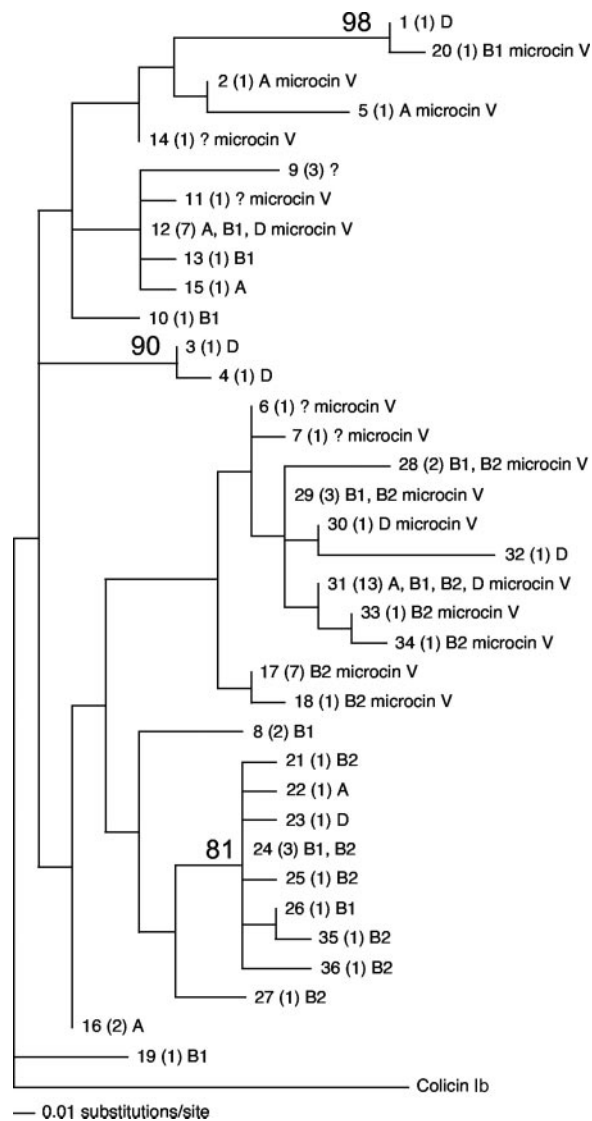


FIG. 1. Maximum-likelihood tree of the 36 Ia nucleotide sequence types detected in *E. coli* isolates from human and nonhuman vertebrates living in Australia. In each label, the first number is the ST and the number in parentheses is the number of times the ST was observed, followed by the genetic groups of *E. coli* in which the ST was detected and finally if the ST co-occurred with the microcin V operon in the same strain. Bootstrap values are based on 500 replicates.

The evidence demonstrates that when these two bacteriocins co-occur in a strain, they are usually encoded on a single plasmid. Plasmids encoding only colicin Ia or only microcin V are most often of different incompatibility groups. In theory, therefore, these plasmids could stably co-exist in a cell. What, then, are the selective forces that result in these two bacteriocin operons becoming encoded on a single plasmid? There are likely two reasons for this observation. First, while a strain can have numerous copies of small plasmids, the presence of large conjugative plasmids increases the metabolic stress on the cell and it can be disadvantageous for a cell to harbor more than one large plasmid (35). Second, there may be circumstances under which the cotransfer of both operons can be

TABLE 3. Nucleotide variation in a 527-bp fragment of the microcin V operon found in this study compared to previously published results (26)<sup>a</sup>

Variant <sup>b</sup>	Nucleotide at position:																																																												
	2	14	42	51	55	76	105	109	110	111	112	113	114	115	116	117	118	119	120	121	122	131	134	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	294	295	323	459																			
AF062847 (1)	C	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A															
H030 (1)	G	A	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A											
AF062845 (1)	G	G	C	C	C	G	A	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A										
AF062849 (1)	G	G	C	C	C	G	A	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A									
AF062848 (1)	G	G	C	C	C	G	A	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A								
H368 (1)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A								
H781 (1)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A							
AF062844 (1)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A						
ECCVAC (52)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A					
B177 (1)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A				
H244 (2)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A		
AF062848 (1)	G	G	T	T	A	T	G	T	A	A	T	G	G	A	A	T	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	C	T	A

<sup>a</sup> Positions 2 to 156 correspond to the 5' flanking region, positions 294 to 323 correspond to the immunity gene, and position 459 corresponds to the toxin gene.  
<sup>b</sup> The number in parentheses is the number of times the variant was observed.

TABLE 4. Population differentiation estimates ( $\Phi_{PT}$ ) to determine the extent to which colicin Ia nucleotide sequence variation depended on the host group from which the strains were isolated or whether the strains also encoded microcin V

Comparison <sup>a</sup>	$\Phi_{PT}$	Probability <sup>b</sup>
Ia animal vs Ia human	0.101	0.040
Ia animal vs IaV animal	0.480	0.001
Ia human vs IaV animal	0.386	0.002
Ia animal vs IaV human	0.292	0.001
Ia human vs IaV human	0.127	0.014
IaV animal vs IaV human	0.129	0.081

<sup>a</sup> Ia, colicin Ia; V, microcin V; IaV, colicin Ia plus microcin V.

<sup>b</sup> The probability that a greater  $\Phi_{PT}$  value could have arisen by chance.

expected to evolve (23). Consider a community consisting of three populations of cells, one producing colicin Ia, one producing microcin V, and a plasmid-free sensitive population. If conjugation moves a microcin V plasmid into a cell harboring a colicin Ia plasmid or vice versa, then the cell will potentially accrue a significant fitness advantage, as it is capable of killing all of the other cells in the community and cannot be killed by any cell. However, if the two operons are maintained on separate plasmids, then it is unlikely that they will always cotransfer to new recipients. Thus, in environments where colicin Ia

and microcin V producers are present, plasmids that encode both bacteriocins should be favored.

Colicin Ia and *E. coli* appear to share a long evolutionary history, while microcin V appears to have arrived in the species relatively recently. For the Australian isolates, the average proportion of polymorphic sites in colicin Ia is 0.0065, significantly less than the average for housekeeping loci in the species (0.013 [unpublished data]), but much greater than the diversity observed for the microcin V operon (0.0005). The GC content of the microcin V operon is 39%, while it is 50% for colicin Ia and 53% for *E. coli* housekeeping genes (unpublished data). Further, the data suggest that in *E. coli*, colicin Ia normally occurs on a plasmid in the absence of microcin V. Overall, colicin Ia occurred 68% of the time on its own; 88% of the time in the isolates from animals, and 43% of the time in the isolates from humans. By contrast, the microcin V operon was detected in the absence of colicin Ia 36% of the time.

The balance of the evidence suggests that the coassociation of colicin Ia and microcin V is a result of the microcin V operon moving onto colicin Ia plasmids. Microcin V plasmids appear to be very homogeneous, while colicin Ia plasmids and plasmids encoding both bacteriocins are diverse (Table 2). However, if this is the direction of operon movement, then a number of genes must cotransfer with the microcin V genes.

TABLE 5. Amino acid sequences of the colicin Ia variants detected in this study together with the human or nonhuman vertebrate sources from which the strains were isolated and whether the strains also encode microcin V

Strain	Source	Microcin V	Variant <sup>a</sup>	Nucleotide at position:																	
				4	60	88	92	109	130	141	169	206	238	241	247	275	284	295	340	346	367
H190	Human	+	3 (1)	R	G	T	I	G	R	R	H	A	A	V	A	N	A	L	A	T	E
B439	Animal	+	6 (1)	R	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	T	E
B313	Animal	+	14 (1)	S	G	T	M	A	R	R	H	A	A	V	A	N	A	L	A	T	E
TA311	Animal	+	16 (1)	S	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	T	E
M550	Animal	+	17 (2)	S	G	T	M	A	R	R	Y	A	A	V	A	N	A	L	A	T	E
AE016	Human	+	4 (1)	R	G	T	M	A	R	R	H	A	A	V	A	N	A	L	A	T	E
H098	Human	+	5 (1)	R	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	S	E
H314	Human	+	6 (7)	R	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	T	E
H151	Human	+	14 (12)	S	G	T	M	A	R	R	H	A	A	V	A	N	A	L	A	T	E
H244	Human	+	15 (1)	S	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	S	E
PE002	Human	+	16 (1)	S	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	T	E
AE008	Human	-	13 (1)	S	G	T	M	A	R	R	H	A	A	V	A	K	A	P	A	T	E
CE015	Human	-	15 (1)	S	G	T	M	A	R	R	H	A	A	V	A	N	A	P	A	S	E
SE017	Human	-	2 (2)	R	G	A	N	G	R	R	H	A	A	V	A	N	A	P	A	S	E
H730	Human	-	10 (2)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	P	A	T	E
H591	Human	-	11 (1)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	P	V	S	E
H556	Human	-	18 (1)	S	G	T	N	G	R	O	H	T	A	V	A	N	A	P	A	T	E
H468	Human	-	19 (1)	S	G	T	N	G	R	R	H	A	A	V	A	N	A	P	A	S	E
H422	Human	-	21 (1)	S	G	T	N	G	R	R	H	T	A	V	A	N	A	P	A	T	E
PE001	Human	+	7 (2)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	L	A	T	E
H331	Human	+	8 (2)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	P	A	S	E
PE004	Human	+	9 (1)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	P	A	S	K
TA060	Animal	-	1 (2)	R	G	A	N	G	H	R	H	A	A	V	A	N	A	P	A	T	E
M411	Animal	-	8 (1)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	P	A	S	E
TA026	Animal	-	10 (3)	R	G	T	N	G	R	R	H	A	A	V	A	N	A	P	A	T	E
R148	Animal	-	12 (1)	R	G	T	N	G	R	R	H	T	A	V	A	N	A	P	A	T	E
R529	Animal	-	20 (1)	S	G	T	N	G	R	R	H	T	A	I	A	N	A	P	A	T	E
TA261	Animal	-	21 (3)	S	G	T	N	G	R	R	H	T	A	V	A	N	A	P	A	T	E
B564	Animal	-	22 (1)	S	G	T	N	G	R	R	H	T	A	V	A	N	T	P	A	T	E
M008	Animal	-	23 (1)	S	G	T	N	G	R	R	H	T	A	V	S	N	A	P	A	T	E
M318	Animal	-	24 (1)	S	G	T	N	G	R	R	H	T	V	V	A	N	A	P	A	T	E
M028	Animal	-	25 (1)	S	V	T	N	G	R	R	H	T	V	V	A	N	A	P	A	T	E

<sup>a</sup> The number in parentheses is the number of times the variant was observed in that source population and in the presence or absence of microcin V.

All plasmids encoding microcin V also encode the virulence factors *iss* and *iroN*, as well as the plasmid transfer gene *traT*. Among 92 strains encoding only colicin Ia, 59% were *traT* positive, 39% were *iroN* positive, and 24% were *iss* positive, but only 12% were positive for all three traits (unpublished data).

One plasmid encoding microcin V has been sequenced in its entirety (17). On the basis of the published plasmid sequence, the transfer of the microcin V genes together with *iss* and *iroNEDCB* would involve a 20-kb DNA fragment. In another plasmid encoding remnants of the microcin V system (18), *iss*, *iroNEDCB*, and the microcin genes are also encompassed by a 20-kb fragment. The inclusion of *traT* with *iss*, *iroNEDCB*, and the microcin V genes would result in a DNA fragment of at least 65 kb. This suggests that microcin V and the associated virulence genes may only transfer to particular plasmid backbones, i.e., those that are *traT* positive. There is additional evidence to suggest that the movement of microcin V is dependent on the genetic background of the colicin Ia plasmid. The majority, 83%, of the stains positive for both colicin Ia and microcin V for which Ia sequence data were available ( $n = 66$ ) were associated with particular amino acid sequence variants (Table 5). The amino acid variants represented 10 nucleotide sequence types, and the average proportion of nucleotide differences among these 10 sequence types was  $0.0021 \pm 0.0008$ .

The association of the microcin V genes with colicin Ia appears to have occurred relatively recently and on multiple occasions. Inspection of Fig. 1 suggests a minimum of six events, but this may represent a minimum estimate. There are 10 STs representing similar amino acid variants of colicin Ia. The hypothesis that these variants may have arisen from a single ancestor is at odds with the observation that virtually no variation has accumulated in the microcin activity and immunity genes, while the Ia toxin gene has accumulated 20 times the number of polymorphisms.

Although encoded on plasmids with the ability to transfer among strains representing all *E. coli* lineages, the presence of microcin V on a plasmid appears to prevent these plasmids from establishing themselves in hosts with particular genetic backgrounds. Why the occurrence of microcin V is incompatible with particular genetic backgrounds is unknown.

Three nonmutually exclusive hypotheses have been suggested to explain why it might be beneficial for a cell to produce multiple types of bacteriocin (10). The first is an expanded killing range. As previously explained, in a community of cells where two types of bacteriocin are being produced, any cell that produces both bacteriocins can kill all single-bacteriocin producers. Second, the evolution of resistance to many bacteriocins is common (29). The production of two or more bacteriocins exploiting different receptors, or different epitopes of the same receptor, may slow the evolution of resistance. Third, multiple-bacteriocin production may confer a fitness advantage in different environments. Colicin Ia is induced via the SOS system during times of stress, while microcin V is induced when iron is limited. Experiments are now under way to determine the advantages to an *E. coli* cell of encoding microcin V and colicin Ia.

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