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Plasmid Genes Required for Microcin B17 Production

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The production of the antibiotic substance microcin B17 (Mcc) is determined by a 3.5-kilobase DNA fragment from plasmid pMccB17. Several Mcc⁻ mutations on plasmid pMccB17 were obtained by both transposon insertion and nitrosoguanidine mutagenesis. Plasmids carrying these mutations were tested for their ability to complement Mcc⁻ insertion or deletion mutations on pMM102 (pMM102 is a pBR322 derivative carrying the region encoding microcin B17). Results from these experiments indicate that at least four plasmid genes are required for microcin production.

Microcin B17 is a hydrophobic peptide of about 4×10^3 to 5×10^3 daltons which inhibits DNA replication in *Escherichia coli*. The inhibition is followed by bacterial DNA degradation and high expression of SOS functions (M. Herrero and F. Moreno, submitted for publication; M. Herrero, R. Kolter, and F. Moreno, submitted for publication). Both production of and immunity to this antibiotic are determined by plasmid pMccB17. This conjugative 70-kilobase (kb) plasmid, which is found in 1 or 2 copies per chromosome, belongs to the incompatibility group FII and does not contain any conventional antibiotic resistance marker (2). The production of microcin B17 is greatest in minimal medium, and, as with other microcins but unlike colicins, it is not inducible by DNA-damaging agents (3).

We are interested in the genetic control of microcin B17 production. In a previous paper we showed that the genetic determinants for both microcin production and microcin immunity are located in a 5.1-kb *Bam*HI-*Eco*RI DNA fragment of pMccB17 (12). This fragment was cloned in pBR322, and the resulting plasmid, named pMM102, directed overproduction of microcin B17. Plasmid mutants unable to produce microcin were obtained from pMM102 by DNA manipulations in vitro and by the insertion of different transposable elements. The study of these mutants showed that the production of microcin B17 specifically required a segment of about 3.5 kb within the 5.1-kb *Bam*HI-*Eco*RI fragment, a quantity of DNA greatly exceeding the quantity required to encode a peptide of 4,000 daltons.

In this paper we present the results of genetic complementation experiments between Mcc⁻ mutants. These results indicate that the 3.5-kb segment contains at least four cistrons, all of them required for microcin B17 production.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and characteristics of strains and plasmids used in this study are listed in Table 1. All genetic manipulations were performed as described previously (12).

Media and antibiotics. Liquid and solid LB medium and minimal M63 medium were prepared as described by Miller (10). Minimal medium was supplemented with glucose (0.2%) and vitamin B_1 (1 μg/ml). Antibiotics were used at the following final concentrations (in micrograms per milliliter): ampicillin (Ap, 40), tetracycline (Tc, 20), kanamycin (Km, 20), streptomycin (Sm, 100), nalidixic acid (Nal, 40).

Nitrosoguanidine mutagenesis and selection of mutants. The mutagenesis procedure with nitrosoguanidine was as described by Adelberg et al. (1). A microcinogenic culture of RYC200.4 growing exponentially was treated with 100 μg of *N*-methyl-*N'*-nitrosoguanidine per ml for 30 min. After mutagenesis, cells were washed twice in phosphate buffer, diluted in fresh LB medium, distributed into several flasks, and incubated overnight to allow segregation of possible mutant plasmids. Samples of the overnight cultures were plated on M63 agar, and the microcin production of the colonies was tested. Those clones that showed an altered microcin production were purified, and single colonies were retested.

Isolation and characterization of pMccB17::Tn*5*. Tn*5* insertions in pMccB17 were obtained from strain RYC820 by selecting for plasmid-mediated transfer of kanamycin resistance into strain BM21. The Km^r Nal^r transconjugants were purified on selective media and then screened for microcin production. Nonmicrocinogenic colonies were further analyzed. Linkage of Tn*5* insertion mutations to pMccB17 was tested by conjugation with pop3351 as the recipient strain. To map the transposon insertions, pMccB17 and pMccB17::Tn*5* DNAs were isolated as described by Clewell and Helinski (6), purified in cesium chloride-ethidium bromide gradients, digested with restriction endonucleases in buffers suggested by the suppliers, and electrophoresed in vertical 0.8% agarose gels in TBE buffer (9). Transposon insertions were located relative to restriction sites of pMccB17 plasmid DNA, taking into account the *Hin*dIII, *Bam*HI, and *Sal*I restriction sites introduced by Tn*5* (8). The physical map of pMccB17 for these enzymes has been described previously (12). Molecular weights of restriction fragments were calculated by comparing relative mobilities of plasmid DNA fragments with those of the *Hin*dIII and *Eco*RI fragments of lambda DNA (11).

Complementation tests. Complementation tests between Mcc⁻ mutations on pMM102 and those on pMccB17 or pMM4 were done in a *recA* strain (RYC819) to prevent homologous recombination between the plasmids. Plasmid pMM102 and its Mcc⁻ derivatives were introduced into RYC819 by transformation selecting for Ap^r. Plasmid pMM4

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Origin or reference
BM21	F⁻ *gyrA* (λ⁺)	7
RYC200.4	BM21(pMM4)	7
pop3351	F⁻ *araD139 ΔlacU169 ΔmalB1 relA1 thi rpsL*	7
RYC100.4	pop3351(pMM4)	7
RYC819	pop3351 *recA56*	This work
RYC820	F⁻*araD139 ΔlacU169 rpsL malE*::Tn*5 relA thiA* (pMccB17)	This work

and its Mcc⁻ derivatives and pMccB17::Tn*5* plasmids were then introduced into the transformants by conjugation selecting for Ap^r Str^r and Tc^r or Km^r. Strains were then stabbed onto a lawn of sensitive cells (RYC819) and checked for microcin production. After overnight incubation, the diameter of the zone of growth inhibition was measured.

RESULTS

Plasmids and the Mcc⁻ mutations. The phenomenon of incompatibility complicates genetic complementation analysis of plasmid-borne mutations. However, to obtain stable diploids, the plasmid DNA region under study may be cloned in a compatible plasmid. Both plasmids can then coexist in the same cell. Mutants impaired in the same function can be selected on the two plasmids and then tested for complementation. This way we have identified the cistrons involved in microcin B17 production.

The pairs of plasmids used were the wild-type plasmid pMccB17 and its derivative pMM4 on the one hand and the compatible hybrid plasmid pMM102 on the other. These three plasmids have been described elsewhere (7, 12). In short, pMM4 is a derivative of pMccB17 which contains a copy of Tn*10* near but not within the microcin B17 genetic region. pMM102 was obtained by cloning the microcin B17 coding region, a 5.1-kb *Bam*HI-*Eco*RI fragment, into the *Bam*HI and *Eco*RI sites of pBR322. This hybrid plasmid

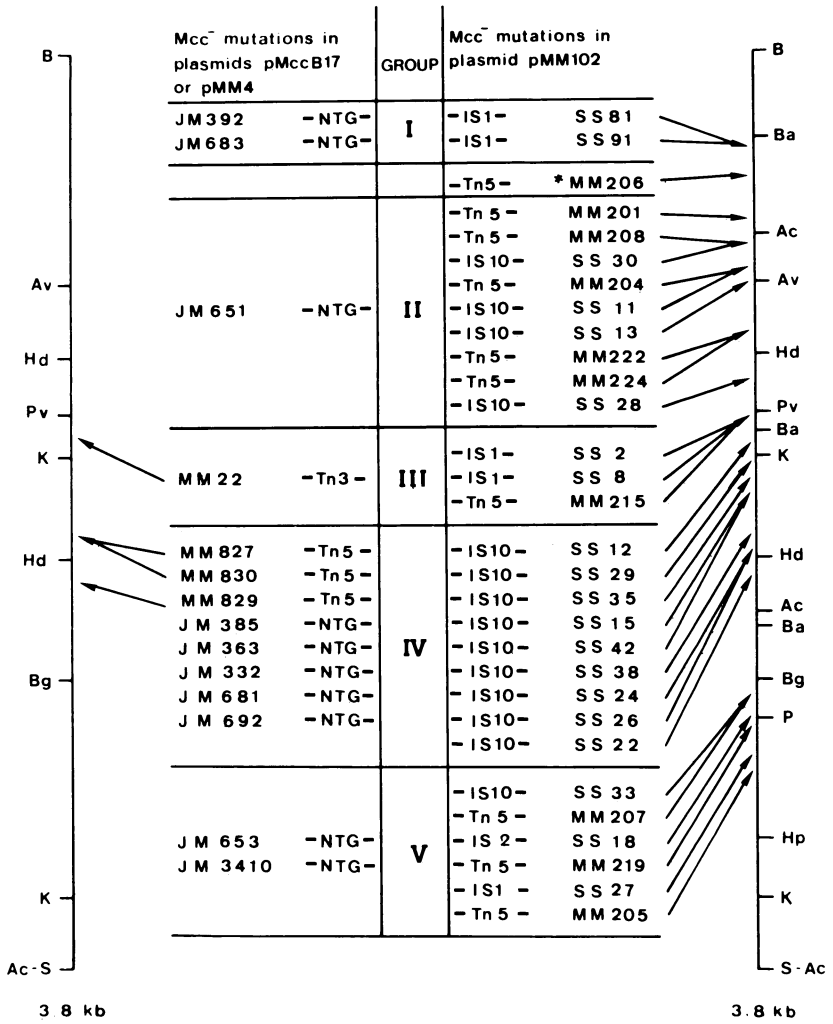

FIG. 1. Mcc⁻ mutations and their arrangement into complementation groups. The nature of each mutation is indicated as follows: NTG, nitrosoguanidine; IS, insertion sequence; Tn, transposon. The physical map of the microcin B17 DNA production region and the location of mutations in pMM102 are from reference 12. For mutations in pMccB17 and pMM4, see the text. I, II, III, IV, and V designate the complementation groups. Symbols: Ac, *Acc*I; Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Hd, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I. *, Mcc⁺.

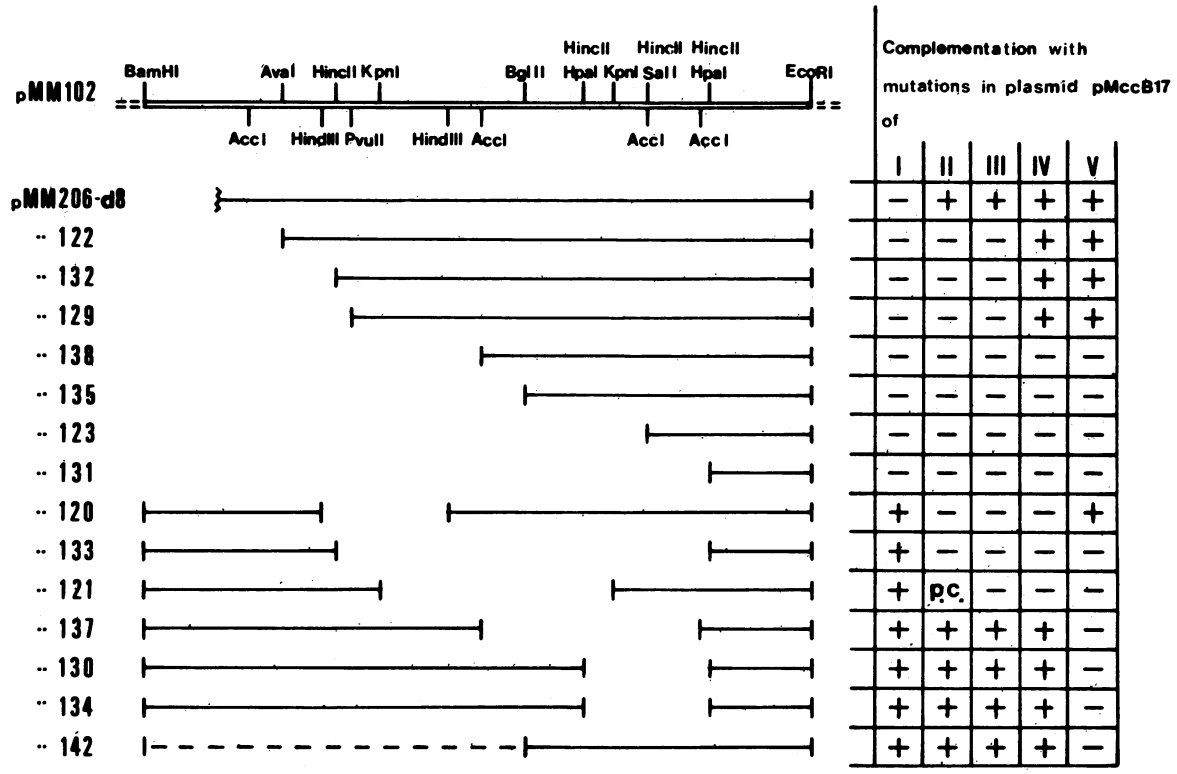

FIG. 2. Physical map and complementation results of deletion mutations of pMM102. The microcin DNA present in the deletion plasmids is indicated by the solid lines. Symbols for complementation are explained in Table 2, footnote *a*.

directs overproduction of microcin and also determines microcin immunity.

The Mcc$^-$ mutants from pMM102 used in the complementation experiments presented below have been described previously (12). The right side of Fig. 1 shows the nature and location of insertion mutations on pMM102. Figure 2 presents the structure of the deletion mutations.

Mcc$^-$ mutations from wild-type pMccB17 were obtained by Tn*5* insertion mutagenesis and characterized as described in Materials and Methods. Among the 18 kanamycin-resistant BM21 clones harboring pMccB17::Tn*5* plasmids, 3 were Mcc$^-$ Imm$^+$. The physical locations of the transposons in these plasmid mutants (MM827, MM829, and MM830) are shown on the left side of Fig. 1.

Mcc$^-$ mutations in plasmid pMM4 were produced by treating strain RYC200.4 with nitrosoguanidine as described in Materials and Methods. From 6,000 colonies tested, 30 mutants with an altered microcin production phenotype were isolated. Plasmids of these clones were transferred to strain pop3351, selecting for Tc^r Str^r transconjugants. Their microcin production was assayed to determine if the mutation responsible for the Mcc$^-$ character was located in the plasmid. Among the 30 mutants, 19 were found to have plasmid borne mutations. However, transconjugants from the other 11 mutants produced microcin as well as strain RYC100.4 did. These mutants were due to chromosomal mutations in RYC200.4, one of which was characterized as being in the *ompR* gene (7). Among the plasmid mutants, two classes could be distinguished. The first class, containing nine mutants, showed decreased microcin production. The second class (left side of Fig. 1), containing the remaining 10 mutants, did not produce microcin at all and were used in the present study. Finally, another Mcc$^-$ mutant used in this work was MM22, which resulted from insertion of Tn*3* into plasmid pMM4. Its isolation and characterization have already been described elsewhere (12).

Complementation experiments. Strains containing pMM102 or this plasmid together with any of the Mcc$^-$ plasmid mutants derived from pMccB17 or pMM4 produced a 2-cm inhibition halo. The diameter of the inhibition halo was 1.5 cm for cells carrying pMM4 or this plasmid together with any of the Mcc$^-$ derivatives of pMM102. These results indicate that all of the Mcc$^-$ mutations studied were recessive.

When plasmid mutants were assayed for complementation, three patterns were observed. Some combinations produced no inhibition halo (no complementation); others produced a halo of inhibition whose diameter ranged from 1.5 to 2 cm (normal complementation); and other combinations produced only small inhibition halos (partial complementation). From the pattern of complementation exhibited by the diploid strains, the 48 mutations obtained by nitrosoguanidine and insertion mutagenesis were arranged into five groups (Table 2). All mutations within a given group do not complement each other, but they complement mutations in other groups. However, mutations in group III also fail to complement mutations in groups II and IV. This pattern of complementation correlates well with the physical location of mutations (Fig. 1). It must be noted, however, that mutations obtained after nitrosoguanidine mutagenesis have not been physically mapped. Notice also that in contrast to the normal complementation of mutations of group IV in pMccB17 or pMM4 by mutations of group II in pMM102, the complementation of mutations of group IV in pMM102 by the only mutation of class II in pMM4 (JM651) is partial.

A second series of complementation experiments was

TABLE 2. Complementation groups established with Mcc point and insertion mutations

pMM102 Group	Complementation[a] of mutations in pMccB17 and pMM4 group				
	I	II	III	IV	V
I	−	+	+	+	+
II	+	−	−	+	+
III	+	−	−	−	+
IV	+	p. c.	−	−	+
V	+	+	+	+	−

[a] + Positive complementation; −, no complementation; p. c., partial complementation.

carried out between deletion mutations in pMM102 and the mutations in pMccB17 and pMM4. Two mutations, MM827 and JM363, were chosen to represent class IV. The results of these experiments as well as the physical structure of deleted plasmids are shown in Fig. 2. As expected, the deleted plasmids, pMM123 and pMM131, failed to complement any Mcc⁻ mutation in pMccB17, since those plasmids had lost the entire microcin production region. The same result was found with plasmids pMM135 and pMM138, which carried only a part of the right side of the production region. Plasmids pMM122, pMM132, and pMM129, which are lacking the left side of the microcin region, do not complement mutations of groups I, II, and III, but they do complement mutations of groups IV and V. Plasmids pMM137, pMM130, and pMM134, which lack only the right side of the microcin production region, complement mutations of groups I, II, III, and IV, but they do not complement mutations of group V. As expected, plasmid pMM120, which has lost 0.8 kb from the center of the microcin region, does not complement mutations of groups II, III, and IV, but it does complement mutations of groups I and V. Plasmid pMM206-d8, which lacks only 0.6 kb from the left of the segment, complements every mutation except those of group I. It is interesting to note that plasmid pMM142, which contains all the DNA present in pMM102 but in which the orientation of the 2.6-kb *Bam*HI-*Bgl*II fragment on the left has been inverted, complements mutations of groups I, II, III, and IV, but it does not complement mutations of group V. Finally, in one case we found partial complementation in the combination involving plasmid pMM121 (Δ*Kpn*I) and the JM651 mutation.

DISCUSSION

In an earlier paper we showed that a 3.5-kb segment of DNA within a 3.8-kb *Bam*HI-*Sal*I DNA fragment from pMccB17 contains all of the plasmid information required to produce microcin (12). As such, the sequence could encode for as much as 125,000 daltons of protein. Since microcin B17 is only about 4,000 daltons, it seemed likely that this region could contain several genes involved in the production of active microcin. The results presented in this paper show that this is indeed the case. Mutations in plasmids pMccB17, pMM4, and pMM102 can be classed in five different groups (I, II, III, IV, and V) according to their behavior in genetic complementation experiments. Each group is composed of mutations not complementing each other, but able to complement mutations of other groups. However, mutations in group III do not complement each other and in addition do not complement mutations of groups II and IV. The simplest interpretation of these results is that groups I, II, IV, and V define four cistrons. This interpretation is supported by the good correlation between functional data and the location of the mutations; i.e., all mutations within a group map within a continuous segment of DNA.

Thus, we conclude that at least four cistrons, A, B, C, and D, defined by complementation groups I, II, IV, and V, respectively, are required for microcin B17 synthesis (Fig. 3). Cistron A is located within the segment of 600 base pairs (bp) limited by the *Bam*HI site and the insertion site of Tn*5* in plasmid pMM206. Cistron B extends from this insertion site to very near the *Kpn*I-1 site, because plasmid pMM120 (Δ*Hin*dIII) is B⁻, whereas plasmid pMM121 (Δ*Kpn*I) is B⁺. Cistron C begins between the *Pvu*II and *Kpn*I-1 sites, since plasmid pMM129 (Δ*Pvu*II) is C⁺, whereas the mutation SS12 located to the left of the *Kpn*I-1 site is B⁺ C⁻. Cistron C must extend to the vicinity of the *Acc*I-2 site, because the C⁻ mutations SS22 and MM829 are located just to the left of this site, but not farther because plasmid pMM137 (Fig. 2) is C⁺. Cistron D probably begins just to the left of the *Acc*I-2 site because plasmid pMM138, which contains all microcin DNA to the right of this site, is D⁻. The right end of cistron D must be located to the right of the *Hpa*I-1 site since plasmid pMM130 (Δ*Hpa*I) is D⁻. It is likely that cistron D ends where the immunity determinants begin, at a point between the *Kpn*I-2 and *Sal*I sites, which is indicated by the phenotype of plasmids pMM121 (Imm⁺) and pMM123 (Imm⁻). In conclusion, the products of four plasmid cistrons of about 300 bp (A), 1,000 bp (B), 900 bp (C), and at least 1,000 bp (D) are required to produce microcin B17. This view of the microcin B17 region is supported by our preliminary results on the products which this region encodes and by DNA sequencing data (R. Kolter, O. Genilloud, and F. Moreno, unpublished results).

What about mutations of group III? All of these are located in a very small segment near the *Pvu*II site; they are not interspersed with those mutations defining cistrons B and C, but they are phenotypically B⁻ C⁻. They could reflect a possible overlapping of cistrons B and C over a short distance, i.e., less than 140 nucleotides (the distance between the *Pvu*II site and mutation SS12). This possibility is suggested by the results of complementation involving MM22 and the deletion mutations of pMM102. Only those derivatives containing the entire B and C cistrons are able to complement mutation MM22. DNA sequencing studies of this region are now in progress and should allow us to solve this problem.

In the experiments reported here, no clear polarity effect

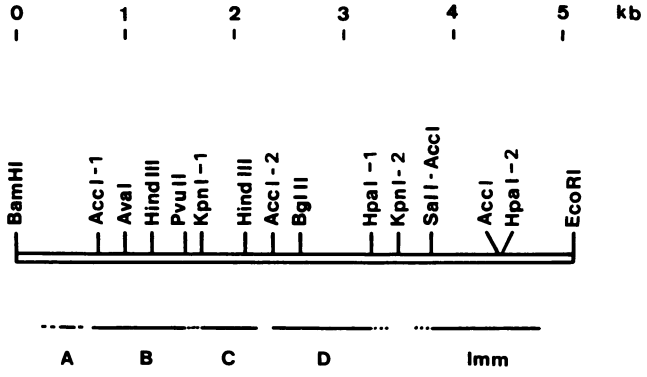

FIG. 3. Genetic organization of microcin B17 region. The location and extent of cistrons required for microcin B17 production (A, B, C, and D) and for immunity expression are indicated.

in the genetic expression was observed, even though we used insertion mutants. This result is particularly interesting because the four microcin cistrons appear to be very close to each other and transcribed in the same direction, from left to right (C. Hernandez-Chico, J. L. San Millan, and F. Moreno, manuscript in preparation), suggesting a typical polycistronic operon structure. Several interpretations can be given for this lack of polarity. First, each gene could have its own promoter. Alternatively, there could be only one promoter on the left end. The complementation results could then be explained if the insertion sequences contain promoters that transcribe through the end of the insert and into the downstream cistrons. This kind of promoter activity has been described for Tn*5* (4) and Tn*10* (5). A promoter located at the extreme left end and transcribing through the entire microcin region was previously proposed to explain the significant reduction of microcin immunity in all insertion mutants and also in those lacking the fragment to the left of the *Ava*I site (12). It is possible that since these genes are actually present in a multicopy plasmid, small amounts of readthrough transcription could be enough to provide sufficient amounts of the needed gene product. Along those lines, it can be argued that JM651 (in plasmid pMM4) is a nonsense mutation in cistron B that reduces the efficiency of translation of cistron C. This possibility could explain its poor efficiency in the complementation of C^- mutations in plasmid pMM102.

The complementation experiments presented here were done in *recA* strains containing derivatives of pMccB17 together with pMM102 or its derivatives. These strains usually overproduce microcin and grow normally in minimal medium. In contrast, plasmid pMM102 alone inhibits the growth of *recA* cells which harbor it. This inhibition, which is complete in minimal medium, is due to microcin production, because Mcc^- pMM102 mutants do not affect the growth of *recA* cells (12). We argued that the overproduction of microcin was not the cause of this inhibition, because *recA* cells containing a low-copy-number minireplicon of pMccB17 in which the microcin region had been recloned also failed to grow. We concluded from those results that plasmid pMM102 lacked genetic information present in wild-type pMccB17 that neutralizes or abolishes any microcin effect when the RecA product is absent. The results presented here confirm this conclusion. Other results indicate that this genetic information, which is different from immunity since pMM102 confers normal immunity, is encoded by DNA sequences adjacent to the immunity region (M. C. Garrido and F. Moreno, unpublished data).

Given the size of the microcin molecule and its peptide nature, it is likely that microcin is a product of translation. Therefore, one of the cistrons identified in these studies is likely to be the structural gene for the microcin. The best candidate for such a gene is cistron A because of its small size (about 300 bp). The products of the other genes could then serve to somehow process and export the product of gene *A*. Work is in progress in our laboratories to identify the role of each of the gene products involved and the pathway leading to production of active microcin B17.

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