Journal of Bacteriology, Dec. 1986, p. 1384–1391
0021-9193/86/121384-08$02.00/0
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Vol. 168, No. 3

Cloning and Mapping of the Genetic Determinants for Microcin C7 Production and Immunity

MARÍA A. NOVOA, LOLA DÍAZ-GUERRA, JOSÉ L. SAN MILLÁN, and FELIPE MORENO*
Unidad de Genética Molecular, Servicio de Microbiología, Hospital "Ramón y Cajal," Madrid 28034, Spain

Received 18 June 1986/Accepted 9 September 1986

Microcin C7, a peptide antibiotic inhibitor of protein synthesis, is produced by *Escherichia coli* K-12 strains that carry the 43-kilobase low-copy-number plasmid pMccC7. Microcin C7 production and immunity determinants of this plasmid have been cloned into the vectors pBR322 and pACYC184. The resulting plasmids overproduce microcin C7 and express immunity against the microcin. Mcc$^-$ and Mcc$^-$ Imm$^-$ mutants have been isolated on recombinant plasmids by inserting transposable elements. Physical and phenotypic characterization of these mutants shows that a DNA region of 5 kilobases is required to produce microcin C7, and that two small regions located inside the producing region are also required to express immunity. Analysis of plasmids carrying *mcc-lacZ* gene fusions indicates that all microcin DNA is transcribed in the same direction. The results suggest that a structure like a polycistronic operon is responsible for microcin C7 production and immunity.

Microcins are dialyzable (i.e., low-molecular-weight) antibiotics produced by enterobacteria (1). Strains producing a specific microcin are immune to the same microcin. As with colicins, both microcin production and immunity are encoded by plasmids, but unlike most colicins, the production of microcins is not inducible by DNA-damaging SOS agents (for a review, see reference 3). Hitherto, five types of microcins have been identified by cross-immunity, biochemical, and genetic criteria.

Most microcins are peptides. This is the case of microcin C7, formerly named microcin 7, which is the sole representative of the immunity group C. Microcin C7 was purified from the supernatants of stationary-phase cultures. Structural analysis showed that this microcin is a linear heptapeptide blocked at both ends. The proposed sequence for the native molecule is acetyl-Met-Arg-Thr-Gly-Asn-Ala-Asp-X, where X represents an unidentified acid labile group which substitutes the terminal aspartic acid (13, 14). Microcin C7 is a bacteriostatic agent that inhibits the growth of enterobacteria phylogenetically close to *Escherichia coli*, apparently by blocking protein synthesis (14).

We are interested in the genetic control of synthesis of microcins. Studies on microcin B17, an inhibitor of DNA synthesis (20, 21), have recently been published (15, 18, 19, 28, 29). In this paper we present the first results on microcin C7. We show the involvement of a medium-size monocopy plasmid in the production of this microcin. The plasmid information required to synthesize this antibiotic and to ensure self-immunity has been mapped within a 5.0-kilobase (kb) region. The genetic organization and expression of this region are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The sources and characteristics of the organisms and plasmids employed are listed in Table 1.

Media and chemicals. Liquid and solid LB medium and M63 minimal medium were prepared essentially as described by Miller (27). Minimal medium was supplemented with glucose (0.2%) and vitamin B1 (1 μg/ml). Antibiotics were used at the following final concentrations: ampicillin, 40 μg/ml; tetracycline, 20 μg/ml; kanamycin, 20 μg/ml; streptomycin, 100 μg/ml; nalidixic acid, 40 μg/ml; chloramphenicol 20 μg/ml. The indicator 5-bromo-4-chloro-3-indolyl-D-galactopyranoside was added to plates at 20 μg/ml.

Microcin C7 production and immunity tests. Microcin production was assayed by stabbing single colonies onto a lawn of sensitive cells. After overnight incubation, the microcin C7-producing colonies had a clear growth inhibition zone around them. To determine the immunity of a strain, a few fresh colonies of a microcin C7-producing strain were suspended in M63 minimal medium to obtain ca. 10^8 cells per ml. Drops of this suspension were placed on the surface of M63 agar plates and allowed to run on a straight line on the agar. When the streaks were dried, the plates were incubated at 37°C for 24 h; then single colonies were cross-streaked, and the plates were incubated. Only microcin-immune cells grew in the immediate vicinity of the producing bacteria.

Plasmid DNA manipulations. Plasmid DNA was usually obtained from alkali-lysed cells. DNA was cut with restriction endonucleases, and DNA fragments were ligated with T4 DNA ligase by using standard recipes. Analysis of DNA fragments was carried out in Tris-borate agarose gels with DNA fragments of known sizes as references. All of these techniques were described previously (26).

Genetic methods. Conjugation was carried out as described by Miller (27). Transformation of competent cells and selection of transformed bacteria with the desired phenotype were done as previously described (28).

Isolation of insertions of transposon in plasmids. Tn*3* transposon insertions in plasmids were isolated by introducing the plasmid into the strain SC18 cultivated at 30°C and selecting for the plasmid-mediated transfer of ampicillin resistance into strain pop3351 as described previously (28). Tn*5* insertions in pMccC7 were isolated by infecting strain MC4100 harboring pMccC7 with phage λ467 and selecting for the plasmid-mediated transfer of kanamycin resistance into strain BM21 as described previously (29). Tn*5* insertions into small multicopy plasmids were obtained by infecting strain RYC1000 carrying the plasmid to be mutagenized with λ467.

* Corresponding author.

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

Strains and phages	Genotype	Reference or source
Strains		
BM21	$F^- gyrA(\lambda^+)$	2
BM7006	Wild type (not K-12)[a]	2
MC4100	F^- *araD139* Δ*lacU169 rpsL relA thiA*	8
pop3351	MC4100 Δ*malB1*	18
SC18	F^- *thr leu*(pSC204)[b]	25
RYC1000	MC4100 Δ*rib-7 recA56 gyrA*	16
POII1681TR	*recA56 ara*::(Mu cts)*3*, Mu dII1681[c]	9
RYC3001.6	MC4100 *malT*::Mu cts	19
RYC128	pop3351 *polA1*[d]	Laboratory collection
Bacteriophage λ467	cI857 *O*am8 *P*am29 *b*221 *rex*::Tn*5*	4

[a] BM7006 is the wild-type isolate producing microcin C7.
[b] pSC204 is a plasmid unable to replicate at 42°C that carries a copy of transposon Tn*3*, which confers resistance to ampicillin (25).
[c] For more information on this strain, see reference 9.
[d] The *polA1* allele was introduced into pop3351 by cotransduction with Tn*10* by using P1 grown on strain SY551 (from M. Syvanen). A Tc^s $PolA^-$ derivative was then isolated by the method of Bochner et al. (5).

After infection, samples were distributed in tubes containing 1 ml of fresh LB medium and incubated for 2 h. Samples of these cultures were spread on LB agar plates containing kanamycin, which were then incubated at 42°C overnight. The colonies were next washed off the plates with a small volume of LB broth. Plasmid DNA from these bacteria was isolated and used to transform strain RYC1000 with selection for resistance to kanamycin. Km^r transformants were checked for microcin production and microcin immunity. Then plasmid DNA was isolated, and the transposon insertion site was mapped by restriction endonuclease analysis.

Isolation of *mccC7-lacZ* gene fusion. *lacZ* gene fusions were obtained by mini Mu dII1681 transposition as described by Castilho et al. (9). pCID104 was introduced into strain POII1681TR by transformation with selection for Cm^r. The transformants were then thermally induced to produce phage particles which were used to infect strain RYC3001.6. Cm^r Km^r transductants were selected at 30°C on LB agar plates containing antibiotics and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside. Blue colonies (Lac^+) were tested for microcin production and immunity, and then their plasmids were physically analyzed. β-Galactosidase activity was assayed on exponentially growing cells in M63 medium and expressed as described by Miller (27).

RESULTS

Characterization of plasmid pMccC7. In a previous paper it was reported that the wild-type microcin C7-producing strain BM7006 was apparently able to transfer this property to *E. coli* K-12 strains after mating (2). To confirm this result we crossed BM7006 Str^s and *E. coli* K-12 MC4100 Str^r on LB agar without selective pressure. Bacteria were then restreaked on the same medium supplemented with streptomycin, and isolated colonies were checked for microcin production. The plasmid content of four colonies, two producers of microcin and two nonproducers, was compared with that of strain BM7006. Whereas nonproducer clones lacked plasmid DNA, a single plasmid species was found in producer MC4100 transconjugants. This plasmid, which was about 40 kb in size, was also found in the wild-type strain together with four more plasmids of about 100, 6.1, 5.6, and 2.5 kb, respectively (results not shown). We concluded that the genetic information for production of microcin C7 is borne by a transmissible plasmid which was called pMccC7. The plasmid also encodes for immunity against the microcin. Like other microcin plasmids, pMccC7 does not contain conventional antibiotic resistance markers but contains all of the information needed for self-transfer because it was transmitted by conjugation to strain BM21 from the above MC4100 transconjugants.

Plasmid DNA from MC4100(pMccC7) was isolated by dye-buoyant density centrifugation and then submitted to restriction analysis. Single and double DNA digests with endonucleases allowed us to construct the physical map shown in Fig. 1. Ambiguities on the location of some restriction sites were resolved by analysis of recombinants containing fragments *Pst*I B and *Hin*dIII C and E in pBR322 (6). According to these data, pMccC7 is 43 kb long.

Since pMccC7 lacks easily selectable markers, we obtained a set of derivative plasmids by insertion of transposon Tn*5* in pMccC7, as indicated in Materials and Methods. Exponentially growing BM21 cells harboring different pMccC7::Tn*5* plasmids (Mcc^+ and Mcc^-) were crossed for 30 min with an excess of pop3351 cells, and Km^r Sm^r transconjugants were selected. In every case the number of colonies was similar, the conjugative transfer frequency being approximately 10^{-5} (calculated as the ratio of the number of transconjugants to the number of donor cells).

To determine the incompatibility group of plasmid pMccC7, pMM501, one of the pMccC7::Tn*5* derivatives, was introduced by conjugation into BM21 cells carrying reference plasmids of different incompatibility groups (FI, FII, I1, H1, H2, C, M, N, and X). In every case, transconjugants containing both plasmids were obtained. They were then grown for 50 generations in drug-free medium. Samples of cultures were plated out, and colonies were checked by replica plating for the presence of the antibiotic resistance characters encoded by the plasmids. We detected segregation of the resistance characters only with plasmid R6K, the reference plasmid of the IncX group (11). Indeed, bacteria retained the Km^r character from pMM501, and most of them had lost the ampicillin resistance encoded by plasmid R6K. To exclude that the Km^r segregants were due to Tn*5* transposition into the *bla* gene of R6K or into the bacterial chromosome we examined the plasmid content of five segregant colonies. All of them harbored only plasmid pMM501. It may be concluded that pMccC7 is a transmissible plasmid belonging to the IncX group. Elimination of pMccC7 by R6K was only achieved when selective pressure in favor of pMM501 was exerted. Similar results were reported by others concerning incompatibility between plasmid R6K and R485, an IncX plasmid of 56 kb (17, 22, 30).

pMccC7 resembles R6K in size (43 and 38 kb, respectively), but unlike R6K, which exists in 12 to 15 copies per cell (24), pMccC7 is present in only 1 or 2 copies per chromosome equivalent. The levels of ampicillin resistance determined by two pMM501::Tn*3* plasmids were quite similar to that determined by Tn*3* derivatives of pMccB17, a plasmid known to be present at one to two copies per chromosome (2, 28). Since ampicillin resistance coded by Tn*3* is proportional to the number of copies of the transposon (32), we concluded that pMccC7 is a low-copy-number plasmid. The same conclusion was obtained by measuring the amount of radioactivity incorporated in the plasmid and chromosomal peaks in cells labeled with the

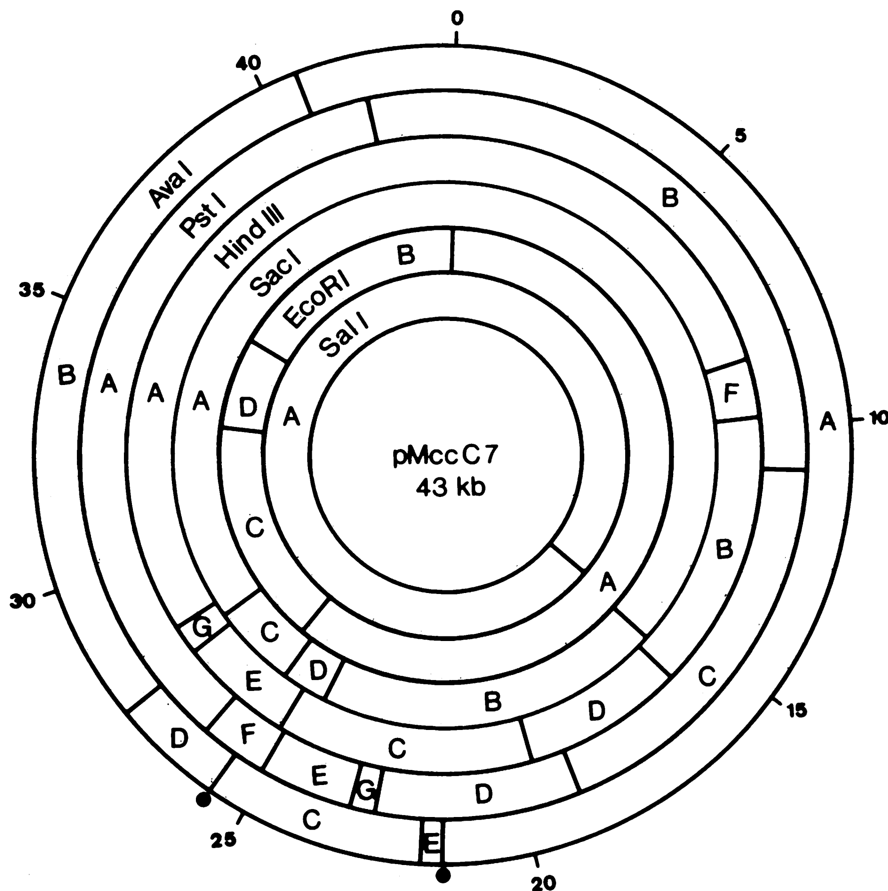

FIG. 1. Physical map of pMccC7. The map was constructed by single and double digests with restriction endonucleases. Microcin determinants are located in *Hin*dIII C and *Hin*dIII E fragments. pMccC7 has no restriction sites for *Bam*HI, *Kpn*I, and *Sph*I. It has seven and nine sites for *Bgl*II and *Hpa*I, respectively. The *Ava*I sites marked with a black circle are *Sma*I sites.

DNA precursor [*methyl*-^{3}H]thymidine (J. C. Pérez Díaz, personal communication). Plasmid R485 has also been reported to be a low-copy-number plasmid (22, 30). When pMM501 was transferred to strain RYC128 the plasmid was stably maintained, showing that, like R6K, R485, and many other large plasmids, pMccC7 is capable of *polA*-independent replication in *E. coli*.

Cloning of microcin C7 determinants of plasmid pMccC7. To identify regions of pMccC7 encoding microcin C7 functions, we carried out two shotgun cloning experiments by using separately the endonucleases *Pst*I and *Hin*dIII and the plasmid pBR322 (6). The ligation mixtures were used to transform strain RYC1000. Transformants were examined for microcin production and microcin immunity. Of a total of 130 clones containing *Pst*I fragments, none was a microcin producer, but three of them were immune to the antibiotic. When the plasmid content of these clones was studied, we found that all three carried the same unique plasmid species of 6.2 kb, which was called pMM560. This plasmid was composed of pBR322 and the 1.85-kb *Pst*I E DNA fragment from pMccC7. The orientation of the cloned fragment within the *bla* gene of pBR322 was the same in all three recombinants.

More than 200 clones containing *Hin*dIII fragments were examined for microcin phenotype. Two of them were microcin immune and microcin overproducers. All others were Mcc^{-} Imm^{-}. Both Mcc^{+} clones harbored the same unique plasmid, which upon *Hin*dIII digestion generated three fragments. One of these was identical in size to *Hin*dIII-cleaved pBR322, and the other two were 5.65 and 2.55 kb long, respectively. The latter ones were the adjacent *Hin*dIII C and *Hin*dIII E fragments from pMccC7. Their relative arrangements in the hybrid plasmid, which was designated as pMM550, was identical to that found in the wild-type pMccC7 (Fig. 1 and 2). The *Hin*dIII C fragment in pMM550 includes the 1.85-kb *Pst*I fragment in pMM560.

Deletion analysis of plasmid pMM550. To obtain preliminary information on the location of the genetic determinants for microcin C7 synthesis on pMM550, we prepared plasmids deleted in vitro for different fragments of the cloned DNA. The structures and phenotypes of these derivatives are shown in Fig. 2, which also includes some constructs in which the orientation of fragments was inverted. The DNA sequences of two *Hin*dIII fragments (C and E) are required to produce microcin and to ensure as much immunity as pMM550. The arrangement of these fragments in pMM550 and pMccC7 had to be maintained to produce the antibiotic and to express immunity, since a plasmid containing the fragments in a different order (pMM553; data not shown) was unable to synthesize microcin. This was confirmed by recloning these fragments from pMM550 in the unique *Hin*dIII site of vector pACYC184 (10). In this case Cm^{r} Tc^{s} transformants were selected and then screened for microcin production and immunity. As expected, all microcin-

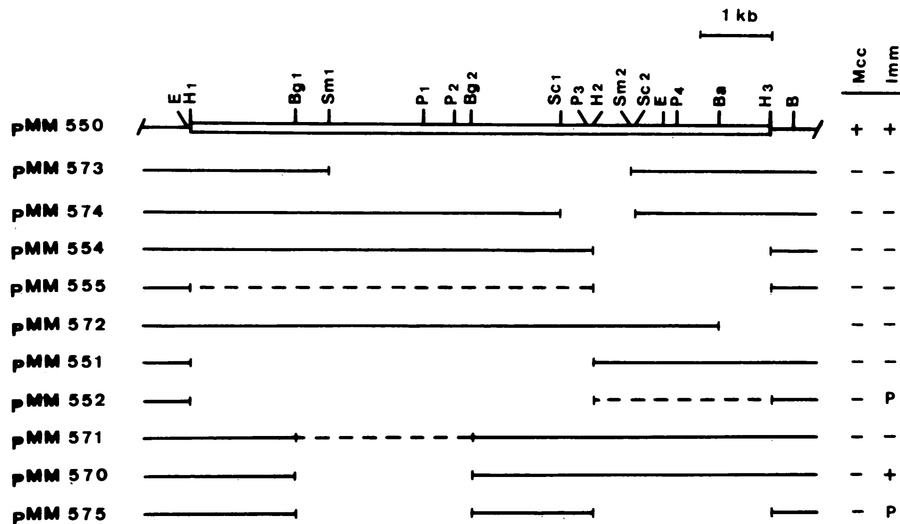

FIG. 2. Physical map and phenotype of deletions in the microcin C7 region. The restriction map of plasmid pMM550 was established by single and double DNA digests. The white box represents DNA from pMccC7 (8,200 base pairs). In pMM550 derivative plasmids solid lines represent sequences known to be present, dashed lines represent those which are inverted, and blank spaces represent those known to be deleted. Abbreviations and symbols: Mcc, microcin production; Imm, microcin immunity; P, immunity is partially expressed; B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*I; Sm, *Sma*I.

producing derivatives were Imm$^+$ and vice versa, and all of them carried the two fragments arranged as in pMM550. One of them, called pCID104, was retained and used for the experiments described below.

Sequences near the right terminus of the smaller *Hin*dIII fragment are also essential for microcin production, since plasmid pMM572 did not produce it at all in spite of possessing all DNA to the left of the *Bal*I site (Fig. 2). DNA sequences located between the two *Bgl*II sites inside the bigger *Hin*dIII fragment are also required to synthesize the antibiotic, since plasmids pMM570 (Δ*Bgl*II) and pMM571 (in which the fragment was inverted) were also Mcc$^-$. The above results indicate that there is genetic information involved in microcin C7 production which extends from near the *Hin*dIII site on the right to beyond the *Bgl*II(2) site on the left.

Results concerning immunity expression by the plasmids in Fig. 2 were paradoxical and are considered below.

Tn*5* mutagenesis of the microcin C7 region of pMM550. Tn*5* transposon insertions into pMM550 were isolated as indicated in Materials and Methods. The insertion sites were mapped with *Bgl*II and *Pst*I endonucleases. These enzymes cut pMM550 in two and five sites and the transposon in two and four sites, respectively; the locations of the sites are

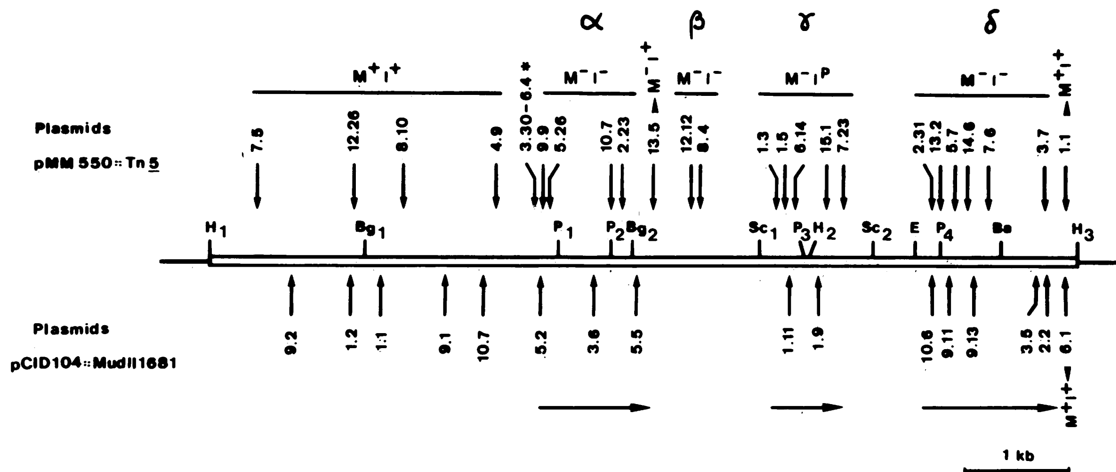

FIG. 3. Mapping and phenotype of insertion mutations in the microcin C7 region of plasmid pMM550. The location of Tn*5* is shown in the upper part of the figure. Tn*5* inserts marked with stars are Imm$^+$ but produce reduced amounts of microcin. Other two independent Tn*5* inserts with the same phenotype mapped in this site. Mutations 15.1 and 7.23 expressed more immunity than the other mutations in region γ. The locations of Mu dII1681s giving in-frame *lacZ* protein fusions are shown in the lower part of the figure. Nine other Mu inserts with the same properties as inserts 9.13 and 3.5 were mapped between these, in region δ. Horizontal arrows under Mu inserts indicate direction of transcription. α, β, γ, and δ designate microcin regions as defined in the text. Abbreviations: M, microcin production; I, microcin immunity. Other abbreviations and symbols are as in Fig. 2.

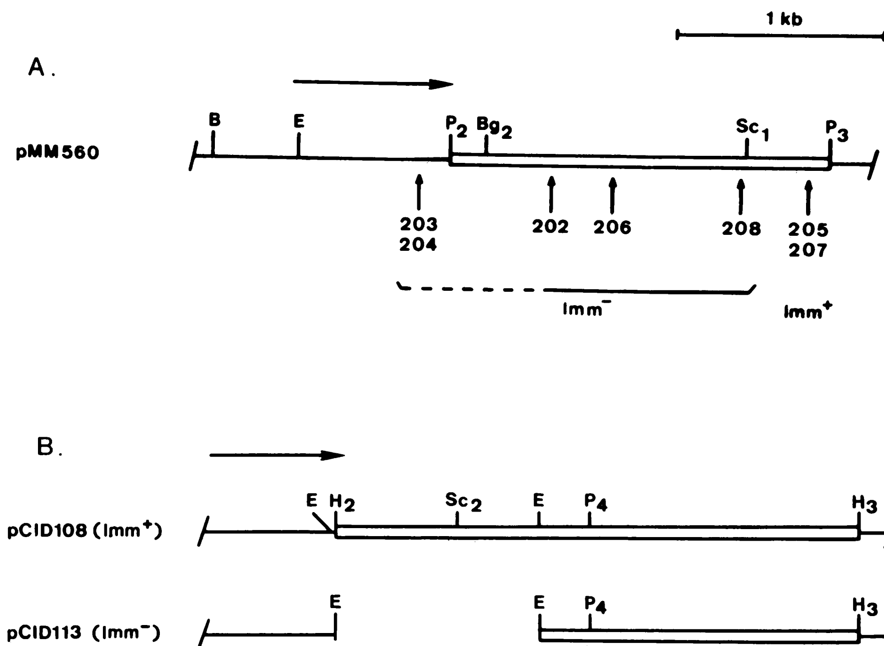

FIG. 4. Recombinant plasmids Imm$^+$ and derivatives. A, Mapping and phenotype of Tn*5* insertions in plasmid pMM560. B, Plasmid pCID108 and its derivative, pCID113. Solid lines represent DNA from vectors (pBR322 in A and pUC8 in B), white boxes represent DNA from pMccC7, and blank spaces represent deleted DNA. Horizontal arrows indicate the direction of transcription from vectors through cloned DNA. Abbreviations are as in Fig. 2 and 3.

known (Fig. 2) (23). Thus, by examining *Pst*I and *Bgl*II digests of mutant plasmids we could deduce the location of Tn*5* insertion sites.

Thirty-seven independent single Tn*5* inserts were mapped and characterized phenotypically (Fig. 3). As expected, insertions mapping within pBR322 DNA did not affect the Mcc$^+$ Imm$^+$ phenotype. The same occurred with five other mutants. Four of them harbored Tn*5* in the *Hin*dIII C fragment from pMccC7, within the 3.2-kb region on the left. Close to vector sequences but on the right end of the *Hin*dIII E fragment mapped the fifth insert that did not affect microcin functions. All the other 28 inserts impaired more or less either microcin C7 production or both immunity and production, and all of them were located along a continuous DNA stretch of 5.0 kb (Fig. 3).

The microcin mutant plasmids could be classified in three groups according to their phenotypes. Mutants in the first group are Imm$^+$ but produced reduced amounts of microcin. The four mutants in this group were indistinguishable by restriction analysis, and inserts were located 200 base pairs apart on the left of *Pst*I(1) site (Fig. 3) in what we suppose to be one of the ends of the microcin C7 region. Mutants in the second group were Mcc$^-$ but expressed a weak immunity; they mapped within a DNA stretch we called γ. Most mutants (the third group) were Mcc$^-$ Imm$^-$, and the mutations were located in three different regions, α, β, and δ (Fig. 3). Although adjacent, α and β were considered different regions because a mutation mapping between them (the Tn*5* insertion 13.5) did not affect immunity, and the mutant produced a very small amount of microcin.

Identification of two microcin C7 immunity determinants. Judging from cross-streak immunity tests, pMM560 determined immunity to microcin C7 as efficiently as wild-type plasmid pMccC7. However, pMM550, which includes the 1.85-kb *Pst*I fragment present in pMM560 (see above), conferred a lower level of immunity. To gain insight on this unexpected result, we prepared new plasmids and looked at the immunity they conferred. First, we mutagenized pMM560 with transposon Tn*5* to map more precisely the immunity determinant carried by this plasmid. We chose seven independent Tn*5* derivatives for analysis. Figure 4A shows the location of the inserts and the phenotype conferred. In three of the Imm$^-$ derivatives the transposon mapped inside the fragment from pMccC7. In the other two, the site of insertion was mapped in the interrupted *bla* gene inside the part proximal to the *bla* promoter. These results indicated that the microcin immunity determinant in pMM560 was expressed by using the *bla* promoter. This was confirmed by inverting the direction of the microcin *Pst*I fragment. The resulting plasmid (pMM561) did not express immunity at all.

Tn*5* insertions within regions α, β, and δ in pMM550 abolished immunity. Region α did not seem to be directly involved in immunity, because plasmid pMM570, obtained by elimination of the *Bgl*II fragment, did not impair the level of immunity encoded by pMM550. Region β is the one cloned in pMM560 which by conferring wild-type immunity defined a first immunity determinant.

The existence of a second immunity determinant in region δ was suggested by the phenotype of the Tn*5* inserts in this region and of the deleted derivative pMM572. It was further substantiated by the fact that plasmids lacking the first determinant could actually confer immunity on their host. In this case the presence of an efficient promoter in front of the determinant was also crucial to the expression of immunity. For instance, whereas no immunity could be detected in cells carrying pMM551, a moderate level of immunity was exhibited by those carrying pMM552 (Fig. 2). In this plasmid, immunity might be expressed from the "antitet" promoter of pBR322, whose transcriptional initiation point is

within the *tet* gene close to the *Hin*dIII site (7, 31). A similar level of immunity was conferred by plasmid pCID111, which harbors the 2.4-kb *Sac*I C fragment from pMccC7 including this immunity determinant (Fig. 1 and 3). This plasmid had been previously obtained by cloning pMccC7 *Sac*I fragments into vector pMM102 (15). When the *Sac*I C fragment was inverted (pCID112) the immunity was completely lost, a result that was expected from our knowledge of the transcription in pMM102 (19). Finally, we cloned the 2.55-kb *Hin*dIII fragment containing this immunity determinant in the *Hin*dIII site of vector pUC8 (33). When the fragment was placed in the proper orientation to be transcribed from the *lac* promoter (pCID108) immunity was expressed as highly as with plasmid pMM560; no immunity could be detected when the fragment was in the opposite direction (pCID109) (Fig. 4B). To map more precisely this determinant, we deleted the *Eco*RI fragment of pCID108. The resulting plasmid (pCID113), which only retained the 1,500-base-pair microcin fragment *Eco*RI-*Hin*dIII(3), was Imm$^-$ (Fig. 4B). This means that the second microcin C7 immunity determinant begins in a point located between sites *Eco*RI and *Sac*I(2), since plasmid pCID111, which only contains DNA sequences on the right of the *Sac*I(2) site, was Imm$^+$ as above indicated.

Isolation of gene fusions into the microcin C7 region. *mcc-lacZ* gene fusions were obtained by mutagenizing plasmid pCID104 with mini Mu dII1681 (see Materials and Methods). As indicated above, pCID104 is a pACYC184 derivative carrying the two *Hin*dIII fragments involved in microcin production. Approximately 35% of the Cm^r Km^r transductants were Mcc$^-$; of these, 15% produced blue colonies on LB plates containing 5-bromo-4-chloro-3-indolyl-D-galactopyranoside. This was close to the expected frequency (17%) for the random insertion of Mini Mu dII transposons given in-frame *lacZ* gene fusions if all DNA or most of it was translated.

A total of 20 independent Lac$^+$ *mccC7-lacZ* gene fusion plasmids were further analyzed. Insertion sites in pCID104 were mapped by digestion with *Bam*HI and *Hin*dIII restriction endonucleases and taking into account the mini Mu dII1681 map in reference 9. When necessary, the location was verified with *Bgl*II or *Sac*I enzyme or both (Fig. 3). All gene fusions inside the microcin region were transcribed in the same direction, that is from the left to the right. As expected, fusions mapping in the α and δ regions were Mcc$^-$ Imm$^-$, and those mapping in the γ region were Mcc$^-$ and partially affected in immunity. No gene fusion mapped in the β region, and every Mcc$^+$ Imm$^+$ fusion mapped outside the microcin region as defined with Tn*5* mutants.

The β-galactosidase activity encoded by fusions was characteristic of each region. Thus, fusions in the α region (for example, 3.6 and 5.5) produced around 4,000 Miller units, fusions in α (1.9 and 1.11) produced 900 units, and fusions in δ (10.6 and 3.5), on the right of the microcin region, produced only 130 units.

DISCUSSION

Microcin C7, a linear heptapeptide which blocks protein biosynthesis in *E. coli* K-12, is produced by wild-type *E. coli* BM7006 (2, 13, 14). Like the other microcin-producing strains, BM7006 is immune to the microcin it produces. We show in this paper that the genetic determinants for microcin C7 production and immunity expression are borne on the 43-kb wild-type plasmid pMccC7. This plasmid has been found to be conjugative and incompatible with plasmid R6K, the prototype of the incompatibility group IncX (11). It is a low-copy-number plasmid with *polA*-independent replication.

Two *Hin*dIII-adjacent fragments of pMccC7, carrying the genetic information to produce microcin C7, were cloned in pBR322. The resulting plasmid, pMM550, overproduced the antibiotic. This plasmid also coded for microcin immunity, but this function was expressed at a lower level than in wild-type pMccC7, a question to be discussed below. DNA insertions (Tn*5*, Mu 1681) within a stretch of 5.0 kb from pMccC7 eliminated the ability of pMM550 to produce microcin. Most plasmids carrying these inserts were also Imm$^-$, and no mutation in this region giving a Mcc$^+$ Imm$^-$ phenotype was found. Thus, Mcc$^-$ Imm$^-$ mutants could be interpreted as resulting from two mutational events, the first being the transposon insertion in a immunity region and the second being a point mutation in a microcin-coding region. In support of this interpretation it might be argued that Mcc$^+$ Imm$^-$ cells should not be viable. However, other results indicate that the Mcc$^-$ Imm$^-$ phenotype was probably due to the insertions that we detected. For instance, when the *Bal*I fragment of pMM550 was deleted in vitro to construct plasmid pMM572, we obtained many transforming colonies, all of them Mcc$^-$ Imm$^-$. Were the deletion to affect an immunity region only, the plasmid would not have been so easily obtained. Moreover, upon reintroduction of the *Bal*I fragment in the proper orientation in pMM572, we recovered both the ability to produce the antibiotic and the immunity expression. This result showed that the microcin region DNA on the left of the *Bal*I site in pMM572 did not carry an Mcc$^-$ (or Imm$^-$) mutation. So, at least this short DNA sequence (500 base pairs) on the right part of the microcin region DNA seems to be actually required for microcin production and for immunity expression. A number of the Mcc$^-$ Imm$^-$ Tn*5* inserts mapped in this sequence. Consequently we assume that most, if not all, Mcc$^-$ Imm$^-$ mutants are actually due to a single mutation.

Considering the phenotype and location of insertion mutations in pMM550 and pCID104, four DNA regions (α, β, γ, and δ) can be distinguished within the 5.0-kb microcin segment (Fig. 3). Insertions in region γ eliminated the ability to produce microcin and decreased to a varied extent the expression of immunity. Region γ is probably required to produce microcin but not for immunity expression; immunity was probably impaired because DNA inserts exerted a polar effect on the expression of region δ, which, as indicated above, is involved in both microcin production and immunity expression. Region α is required for producing microcin but not for expressing immunity, because plasmid pMM570 (Δ*Bgl*II) was as immune as pMM550. Most probably Tn*5* insertions in this region abolished immunity because the transposon exerted a polar effect on the expression of region β and perhaps δ. Region β, or part of it, is required for pMM550 to express immunity. This region must extend through the *Sac*I(1) site because plasmid pMM574 (Δ*Sac*I) was Imm$^-$ (Fig. 2 and 4A). Whether region β is necessary for microcin production is a open question, because the Mcc$^-$ phenotype of Tn*5* insertions in this region may be due to a polar effect of transposons on the expression of region γ or δ or both. This view about the individuality and the role of these different regions agrees with the following facts: (i) all microcin DNA was transcribed from left to right; (ii) β-galactosidase activity synthesized from *mcc-lacZ* gene fusions varied in amount depending on the region in which the fusion joint was located (see Results); (iii) region β encoded for immunity when a strong promoter was provided on its

left side (pMM560) but not when a strong promoter was provided on its right side (pMM561); (iv) the immunity encoded by pMM560 was abolished when Tn*5* was inserted between the immunity determinant and the *bla* promoter; (v) like region β, region δ alone encoded for a high level of immunity when properly oriented behind a strong promoter; (vi) the inversion of the *Bgl*II fragment (i.e., region α) in pMM550 (plasmid pMM571) caused loss of expression of immunity. Since plasmid pMM571 carries complete β and δ regions ordered as in pMM550 and pMM570, these results also indicate that the microcin region or part of it is expressed in pMM550 by using a promoter located either within the *Bgl*II fragment or to its left.

Consequently, all of our results suggest that the production of microcin C7 requires the activity of several adjacent genes (each region may contain one or more genes) located within a DNA segment of 5 kb. These genes seem to be organized as a polycistronic operon structure whose promoter is presumably located on the left side of the *Pst*I(1) site (Fig. 2).

Our results concerning microcin C7 immunity are rather surprising. Multicopy plasmids such as pMM550 and pCID104, which overproduce the antibiotic, confer less immunity than the low-copy-number plasmid pMccC7. In addition, the activities of two determinants borne by separate regions, β and δ, are required for pMM550 to express immunity. However, any of these determinants by itself gives a very high level of immunity when expressed from strong promoters such as *bla* and *lac*. This dependence of the strength of the promoter was widely illustrated in Results. Whereas plasmids pMM554 and pMM555, carrying the determinant in the β region, did not express immunity at all, pMM575 did express a low but significant level of immunity (Fig. 2). This was likely due to the connection of the immunity determinant in β to a stronger promoter located on the left side of the *Bgl*II(1) site. It remains to be explained why the multicopy plasmids pMM550 and pCID104, which overproduce microcin, confer less immunity than pMccC7. This situation resembles that of tetracycline resistance encoded by transposon Tn*10*, which is expressed less when the transposon is present in a high copy number (12). It is possible that overexpression of both immunity determinants, together in the same cell, leads to a decrease in the physiological expression of immunity as a whole. Alternatively, these hybrid plasmids may lack some genetic information that ensures a higher level of immunity from pMccC7. This question is being investigated.

The genetic system described in this work has several points in common with that coding for microcin B17 production and immunity. This last system also lies on a DNA segment of 5 kb, and four adjacent genes have been shown to be required to produce microcin B17 (28, 29). Three genes are involved in the immunity expression, and two of them are also required to produce the antibiotic (M.C. Garrido et al., manuscript in preparation). One of the most intriguing points emerging from results described here and from our studies on microcin B17 is the involvement of the same gene product(s) in both the production of microcin and its immunity. We postulate that these products can intervene in the export of antibiotic outside cells. In this way bacteria could ensure production (output of the antibiotic) and protection against the microcin coming into cells. The existence of such an immunity mechanism does not preclude the existence of a other mechanisms working at other levels, for instance, by protecting the site of action of the antibiotic. This is probably what happens with microcin B17 (our unpublished results). It would be of interest to know whether other microcin genetic systems are as complex as these and whether they are organized according to the same model.

ACKNOWLEDGMENTS

We thank J. Talavera for technical assistance and the reviewers for constructive comments.

This work was supported by grants from Comisión Asesora de Investigación Científica y Técnica (Ministerio de Educación) and Fondo de Investigaciones Sanitarias de la Seguridad Social (Ministerio de Sanidad), Spain.

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