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Microcin H47, a Chromosome-Encoded Microcin Antibiotic of *Escherichia coli*

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Microcin H47 (MccH47) is a novel microcin antibiotic produced by a natural *Escherichia coli* isolate. In contrast to all the other colicins and microcins examined to date, which are plasmid encoded, the genes for MccH47 synthesis and immunity are located on the chromosome. These genetic determinants were cloned and shown to extend over a continuous DNA region of ca. 10 kb.

Many enterobacterial strains produce antibiotic substances that are active on other enterobacteria. These substances have been classified into two groups, colicins (large polypeptides) and microcins (small peptides). Strains producing colicins or microcins are immune (insensitive) to the antibiotics they produce (2, 17). Colicins are bactericidal proteins with a molecular size ranging from 30,000 to 80,000 Da, whose production is induced by agents or conditions that induce the SOS response (16). Microcins, which may be bactericidal or bacteriostatic, are smaller than 6,000 Da, and they are only produced during the stationary phase (2, 7, 11).

Several genetic systems encoding colicins or microcins have been extensively characterized. In all cases the genetic determinants were found to be located on plasmids. The best-studied colicin systems consist of three genes that code for the colicin, the immunity protein, and a small protein involved in the release of the colicin to the extracellular medium (16, 17). Microcin systems are more complex. The microcin B17 system consists of seven genes, four required for antibiotic synthesis, two required for its secretion, and one required for immunity (8, 9). At least six plasmid genes have been identified as being involved in microcin C7 production and immunity (15; L. Díaz-Guerra and F. Moreno, unpublished results). These microcin genes are clustered and extend over a DNA region of 5 to 6 kb.

Using *E. coli* K-12 BZB1011 *gyrA* as the indicator strain, we screened a collection of gram-negative bacteria from different natural sources in Uruguay for the production of novel antibiotics. Because we were mainly interested in microcins, the ability of these substances to pass through a cellophane membrane was checked in order to exclude colicins (1). Eight isolates producing microcinlike activities were identified. One of these (*E. coli* H47), obtained from human feces, was studied further.

When assayed by the patch test, *E. coli* H47 produced clear inhibition halos on strain BZB1011 grown in M63 minimal medium. The addition of isoleucine (40 μg/ml) or mitomycin (0.5 μg/ml) to the plates did not modify the size or the aspect of the halos, but they were greatly reduced in size when the patch test was performed on LB plates. These features are typical of microcins as described by Baquero and Moreno (2). Strain H47 was not susceptible to the activity it produced.

The antibiotic produced by strain H47 inhibited the growth of all *E. coli* K-12 strains carrying known colicin or microcin plasmids (2, 17). The same occurred when BZB1011(pEX3), an *E. coli* K-12 strain bearing the H47 genes (see below), was used as the producing strain. In addition, BZB1011(pEX3) was susceptible to all known colicins and microcins. Therefore, the antibiotic activity produced by the wild-type strain H47 is a novel microcin which defines a new immunity group (H). We call this activity microcin H47 (MccH47). MccH47 was shown to be active on many wild isolates of *E. coli*, *Salmonella*, *Enterobacter*, *Shigella*, *Klebsiella*, and *Proteus* spp. but not on gram-positive isolates of *Streptococcus*, *Staphylococcus*, and *Bacillus* spp.

Since all the known enterobacterial genetic systems encoding antibiotics are plasmid linked, we began the search for the MccH47 determinants by analyzing plasmids carried by strain H47, which contains a small high-copy-number plasmid and a large low-copy-number plasmid (data not shown). H47 was found to be resistant to streptomycin (100 μg/ml), but neither Nal^r Sm^r nor immune MccH47$^+$ Nal^r clones were obtained when *E. coli* K-12 IGOR100 (*hsdR* Nal^r) was crossed with H47 or transformed with its plasmids. This suggested that neither the Sm^r marker nor the MccH47 determinants were located on either of the two plasmids.

Mu d5005 is a mini-Mu plasmid containing the pMB1 replicon and the Km^r marker from Tn*5* and is useful in preparing in vivo DNA libraries (10). We used it to prepare a library from H47, with which strain IGOR103 (*hsdR met* Mu *c*ts Ap^r) was infected at a low multiplicity (0.001). Samples from the infection mixtures were seeded onto M63 plates supplemented with methionine, ampicillin, and kanamycin. After 72 h of incubation at 30°C, the plates exhibited a confluent bacterial growth with some clear areas (antibiosis halos) centered around a colony. Sixteen of these colonies were purified on LB plates containing kanamycin and then assayed for antibiotic production with BZB1011 as the indicator strain. All of them produced large halos of growth inhibition. They were also shown to be cross-immune and immune to the activity produced by the wild-type strain H47. As expected, the size of the halos was larger on minimal medium than on rich medium, and the antibiotic diffused through cellophane membranes. The halos were also larger

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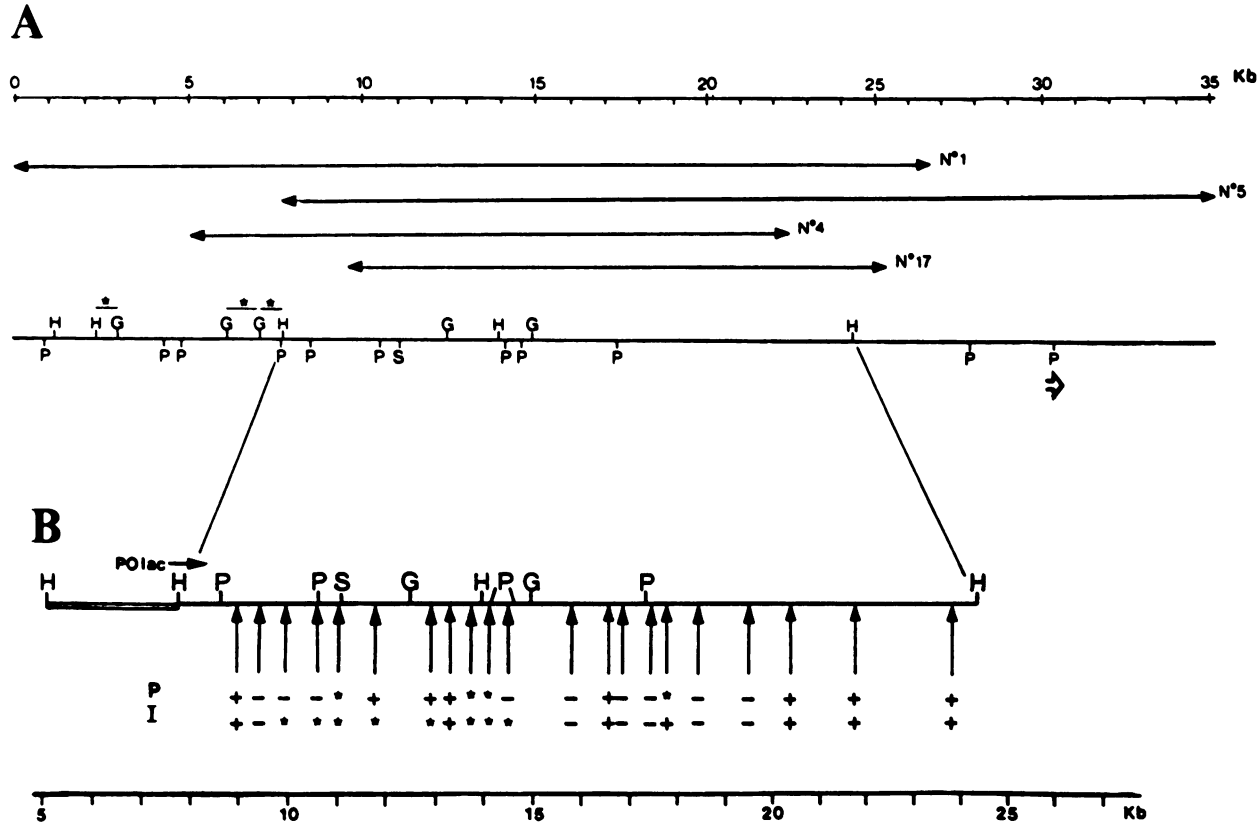

FIG. 1. (A) Physical map of the MccH47 region. Several hybrid mini-Mu d5005 derivative plasmids are represented (numbered 1, 5, 4, and 17). Their physical maps have been overlapped. Restriction sites: H, *Hin*dIII; G, *Bgl*II; S, *Sal*I; P, *Pst*I. The open arrow points to a region containing several *Pst*I and *Bgl*II sites that could not be mapped precisely. The restriction fragments labeled with asterisks were the probes used in the Southern experiment of Fig. 2C. (B) Tn*5* insertions in plasmid pEX4. The single line represents cloned DNA, and the double line represents vector DNA. The arrows indicate the sites of Tn*5* insertions. Mutant phenotypes are indicated below for antibiotic production (P) and immunity (I). Asterisks indicate a partial phenotype. POlac, *lac* promoter-operator.

than those produced by strain H47, indicating that the *E. coli* K-12 derivative clones overproduced the antibiotic.

Plasmids from some of these clones were purified and physically analyzed with several restriction enzymes. Their restriction patterns were compared, and a preliminary physical map of the cloned region was constructed. The sizes of the cloned fragments ranged from 16 to 27 kb, and a minimal common stretch of 13 kb was found in all of them. Therefore, the MccH47 genetic system must be contained in this common fragment (Fig. 1A).

This common region contained a single *Hin*dIII target and was flanked by two other *Hin*dIII sites. To confirm that the H47 genetic determinants were actually located within this region, both *Hin*dIII fragments (6.2 and 10.4 kb) were subcloned together into pUC13 (14). Several Ap^r transformants were obtained, which, like the Mu d5005 derivatives, overproduced the antibiotic and were immune to microcin produced by H47. Some of them grew on minimal medium, but others did not. Plasmids from two clones, one of each class, were purified and physically analyzed. Both plasmids contained the two *Hin*dIII fragments in the correct relative position, but the inserts were differently oriented in relation to the *lac* promoter of the vector (Fig. 1B). Cells harboring pEX3 but not pEX4 grew on M63 medium.

To define the limits of the region involved in microcin functions (production and immunity), we isolated and characterized Tn*5* insertions on plasmid pEX4 by using λ467 *c*I857 *rex*::Tn*5* *O*am29 *P*am80 *b*221 as described before (3, 15). The sites of the insertions and the resulting phenotypes are shown in Fig. 1B. The results indicated that a continuous DNA region of about 10 kb was required to express the antibiotic functions. The two Tn*5* insertions inside the 10-kb stretch that affected neither production nor immunity are probably located in intergenic spaces. Mutants not producing antibiotic grew in M63 medium, while those producing MccH47 did not. We do not know what this correlation between antibiotic production and lack of growth for cells carrying pEX4 derivatives means; notice that cells carrying pEX3, which apparently produced as much microcin as those carrying pEX4, grew in M63. It appears that the genetic system encoding MccH47 (production, secretion, and immunity) exhibits a higher degree of complexity than those encoding microcins B17 and C7 (8, 9, 15).

The region located to the left of the internal *Sal*I site (Fig. 1B) did not seem to be required for immunity expression, since a pEX4 derivative deleted for this region encoded normal levels of immunity. However, the fact that Tn*5* insertions mapping to the left of the *Sal*I site affected the immunity suggests that the entire system may be expressed from a promoter located on the left of this insertion site.

To determine the origin of the cloned DNA region containing the MccH47 system, plasmid and genomic DNAs

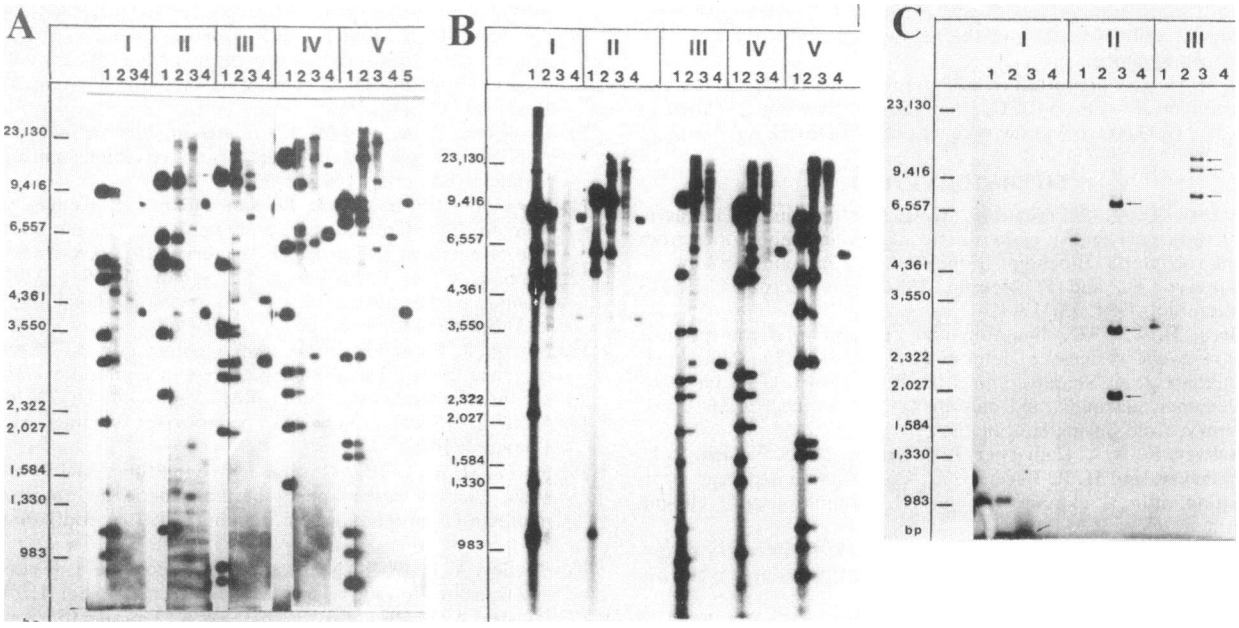

FIG. 2. Hybridization patterns of the MccH47 region. Plasmid DNA was prepared and manipulated as described before (12); total genomic DNA was prepared as in reference 18. DNA samples were electrophoresed though 0.8% agarose slab gels, transferred to Hybond-N (Amersham) nylon filters, and hybridized as previously described (19). Probes were labeled by nick translation (Boehringer kit) with [^{32}P]dCTP. From 50 to 150 ng of labeled DNA (1×10^7 to 3×10^7 cpm) was used per assay. Kodak X-Omat AR X-ray film was used for autoradiography. Probes were *Hin*dII-*Bgl*II digests of Mu d5005 1 (A) and 5 (B) and fragments labeled with asterisks in Fig. 1A (C). DNAs used to probe were digested with *Hin*dIII plus *Bgl*II (I), *Hin*dIII (II), *Pst*I (III), *Eco*RI (IV), and *Sma*I (V). Lane 1, DNA from probes (except in panel C, where DNA was a Mu d5005 derivative Mcc$^+$, not shown in Fig. 1A); lane 2, total DNA from H47; lane 3, chromosomal DNA from BZB1011; lane 4, plasmids purified in a cesium chloride-ethidium bromide gradient from H47; lane 5, as in lane 4, but DNA was not digested. The arrows point to BZB1011 fragments hybridizing with the probe close to the MccH47 region. Sizes are shown at the left (in base pairs).

from strain H47 were purified and digested with several restriction enzymes. DNA fragments were separated on agarose gels and examined by Southern blot hybridization (19). The probes were Mu d5005 derivatives 1 and 5 (Fig. 1A) doubly digested with *Hin*dIII and *Bgl*II. The fragments hybridizing with themselves (Fig. 2A and B, control lanes 1) also hybridized with fragments from the chromosomal DNA of H47 (lanes 2), but they did not hybridize with fragments from H47 plasmid DNA (lanes 4). Depending on the digestions, one or two fragments from H47 plasmid DNA hybridized with the probes, but these fragments were not detected in the digests from the Mu derivatives or from the chromosomal DNA. These plasmid fragments also hybridized with pBR328 DNA when it was used as the probe (data not shown). As pBR328 and Mu d5005 both have the pMB1 replicon (4, 10), this positive reaction indicates that the small high-copy-number plasmid from strain H47 is related to pMB1.

As shown in Fig. 2B (lanes 3), the Mu derivative 5 probe did not detect any fragment from the genomic DNA of *E. coli* K-12 strain BZB1011. However, the probe Mu derivative 1 did hybridize with BZB1011 DNA (Fig. 2A, lanes 3), but the fragments showing a positive reaction did not migrate like the control probe fragments (lanes 1). These positive hybridization patterns were analyzed further with *Hin*dIII-*Bgl*II fragments from the left end of the cloned DNA (indicated by an asterisk in Fig. 1A) as the probe (Fig. 2C). The restriction pattern of this region was recovered in the H47 chromosomal DNA (lanes 2), while the H47 plasmid DNA gave a negative result (lanes 4). *Hin*dIII and *Pst*I digests of BZB1011 chromosomal DNA (lanes 3) yielded three bands of 6.9, 2.8, and 1.9 kb and 11.6, 9.4, and 7.7 kb, respectively, that hybridized with the probe. A band of 0.65 kb from the *Hin*dIII-*Bgl*II BZB1011 digests hybridized with the probe. Indeed, this was the size of the two fragments used as the probe. These results clearly show that the MccH47 determinants are not located on the plasmids we have purified from strain H47. They also indicate that a sequence situated at the left of the microcin region is present in at least three copies in the chromosome of BZB1011. It seems very likely that this sequence is part of an insertion sequence present in several copies in *E. coli* K-12 and in single copy in H47.

Although the possibility that an undetected giant plasmid carries the MccH47 system cannot be totally excluded, the results described above strongly support the conclusion that MccH47 is chromosomally encoded. At this point it may be added that the large plasmid systematically present in our plasmid preparations from H47, was about 100 kb long (data not shown). Therefore, we think we have identified, for the first time, a chromosomal genetic system encoding an antibiotic in a member of the family *Enterobacteriaceae*. Curiously, the opposite situation is found in *Streptomyces* spp.; the genetic determinants coding for antibiotics are located in the chromosome except that coding for methylenomycin production, which is located on a plasmid (5, 13). Finally, we believe that other microcins and colicins, such as microcin E492 from *Klebsiella pneumoniae* and colicin G from *E. coli*, are chromosomally encoded, since all efforts to identify their determinants on plasmids have so far failed (6; J. Blázquez and F. Moreno, unpublished results).

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