Genetic Analysis of Microcin H47 Antibiotic System

CARINA GAGGERO,1 FELIPE MORENO,2 AND MAGELA LAVIÑA1*

División Biología Molecular, Unidad Asociada a la Facultad de Ciencias, Instituto de Investigaciones Biológicas Clemente Estable, Avenida Italia 3318, 11000 Montevideo, Uruguay,1 and Unidad de Genética Molecular, Hospital Ramón y Cajal, Madrid 28034, Spain2

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The microcin H47 genetic determinants span a DNA region of ca. 10 kb and represent the first description of an enterobacterial antibiotic system located in the chromosome of the producing strain. Transcriptional and translational fusions to lacZ showed a complex transcriptional organization of the microcin H47 system. Complementation tests identified six genes that are necessary for the production of the antibiotic; the products of two of them are involved in the export of microcin to the extracellular medium. The immunity determinant was located in an 0.8-kb DNA fragment. There is a putative "silent region" of ca. 3 kb inside the system that could not be clearly related to any antibiotic function. Protein products were identified and assigned to three production genes and also to a gene from the silent region.

Microcins are a group of low-molecular-weight peptide antibiotics produced by members of the family Enterobacteriaceae (1, 2). Microcin H47 (MccH47) was identified as an antibiotic activity that was produced by a natural Escherichia coli isolate from human feces and that inhibited the growth of E. coli K-12 and several enterobacterial strains. In contrast to previously studied microcins, which are plasmid encoded, MccH47 genetic determinants are located in the chromosome of the producing strain. The entire genetic system for MccH47 was cloned and shown to span ca. 10 kb. When introduced in an E. coli K-12 strain, the cloned DNA directs microcin production and immunity and must therefore contain the genetic determinants for these antibiotic functions (16).

The genetic systems of microcins B17 and C7 and colicin V (which is a microcin despite its name) are the most extensively characterized. The microcin B17 system consists of seven contiguous genes transcribed in the same direction and spanning a DNA region of ca. 5.5 kb. The structural gene codes for a precursor of the antibiotic; the products of these genes are required for several posttranslational modifications not yet well characterized; two genes are dedicated to the export of the antibiotic to the extracellular medium; and one gene is required for immunity (8, 9, 14). The colicin V production and immunity genes span 4.5 kb and are arranged in two converging operons. The colicin structural and immunity genes constitute one operon, and the other operon codes for two proteins that are involved in the export of the antibiotic. The product of the unlinked chromosomal gene tolC is also required for colicin V export, a secretion process that is independent of a signal sequence and that is mediated by a multidrug resistance-like export system (10, 11, 14). The information required for microcin C7 production and immunity is encoded in 5 kb of DNA for which four regions transcribed in the same direction have been genetically defined (23).

This article presents the overall genetic organization of the MccH47 antibiotic system. Directions of transcription for the system were established. At least six genes were shown to be necessary for the synthesis and export of MccH47. The region conferring the immunity phenotype was located. Several MccH47 gene products were identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The sources and characteristics of the strains, plasmids, and bacteriophages used in this study are listed in Table 1.

Culture media, enzymes, and chemicals. The media used, LB rich medium and M63 glucose minimal medium, were as described previously (21). Antibiotics were used at the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 20 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was added to solid media at 20 μg/ml. L-[35S]methionine (1,000 Ci/mmole) was purchased from New England Nuclear. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals and New England Biolabs, respectively. Prestained Rainbow protein molecular weight standards were obtained from Amersham.

Microcin production and immunity assays. Microcin production was assayed on M63 glucose medium by a patch test as described previously (24). After overnight incubation at 37°C, an inhibition halo of about 15 mm in diameter was observed when the producing bacteria carried plasmid pEX4 or pEX100. BZB1011 was used as the sensitive indicator strain. Microcin sensitivity was also assayed by a patch test or by a cross-streaking assay as described previously (24). RYC1000(pEX4) or RYC1000(pEX100) was used as the producing strain. The cross-streaking assay was always performed on LB medium.

Some mutant clones showed partial phenotypes of production and immunity, that is, they produced inhibition halos less than 8 mm in diameter and exhibited an intermediate level of MccH47 immunity compared with those of control strains RYC1000(pACYC184) and RYC1000(pEX100).

DNA manipulations. Plasmid DNA was isolated by the alkaline lysis procedure (4). pEX4 and pUC13 were purified in cesium chloride-ethidium bromide gradients. Digestions with restriction endonucleases, ligation with T4 DNA ligase, transformation of competent cells, and agarose gel electrophoresis were performed as described previously (25).

Transposon insertion mutageneses of pEX100. Mud1681
insertion mutagenesis was performed essentially as described previously (6). Strain POI1681 was transformed with pEX100 and then used to produce transducing particles. Strain BZBI011 Mucts Ap was infected with the lysate, and the transduction mixture was incubated for 3 h at 30°C to allow the expression of kanamycin resistance and then was plated on LB agar containing kanamycin, chloramphenicol, and X-Gal. Nine experiments were done to obtain independentinsertions. Mutant plasmid DNAs were isolated and transformed into RYC1000 Mucts Km.

TnlacZ insertion mutagenesis of pEX100 was performed essentially as described previously (18). CC170 competent cells were transformed with pEX100, and eight transformant colonies were purified in LB medium containing chloramphenicol. A colony from each clone was resuspended in 1 ml of LB medium, and then 0.3 ml was plated on LB medium containing chloramphenicol, kanamycin at 300 μg/ml, and X-Gal. The concentration of kanamycin was 10 times higher than usual to favor the growth of cells carrying plasmids with transposon insertions. The plates were incubated for 2 days at 37°C, and confluent growth with overgrowing colonies was obtained. Cells pooled from each plate were resuspended in 5 ml of LB medium, and plasmid DNA was prepared and used to transform CC118. Selection for growth was done on LB medium containing chloramphenicol, kanamycin, and X-Gal. From each experiment, seven blue colonies and one white colony were purified. Plasmid DNA was prepared from each purified clone and used to transform RYC1000 cells.

Complementation tests. Complementation tests with non-producing derivatives of both pEX4 and pEX100 were done by use of a recA strain (RYC1000). Cells already harboring one plasmid were transformed with other compatible plasmids, and selection was performed on LB medium containing ampicillin, chloramphenicol, and kanamycin. From each transformation, three or four colonies were purified and tested for MccH47 production. Positive complementation was judged as the ability of the two compatible plasmids—present together in the same cell—to restore the MccH47 production phenotype.

Detection of MccH47 intracellularly. RYC1000 cells bearing nonproducing mutant plasmids affected in different genes of the system, as well as controls [RYC1000(pACYC184), RYC1000(pEX100), and RYC1000(pEX4)], were lysed by the protocol of Holmes and Quigley (13) with modifications. In brief, cells were grown in M63 glucose medium with antibiotics to avoid plasmid loss until they reached an optical density at 600 nm of 0.5; 16 ml was centrifuged, and the pellet was resuspended in 100 μl of STET (0.1 M NaCl, 10 mM Tris · HCl [pH 8.0], 1 mM EDTA [pH 8.0], 5% Triton X-100). Lysozyme was then added, and tubes were transferred to a boiling water bath for 1 min. After centrifugation and removal of the pellet, MccH47 activity in the supernatant was tested and quantified by the critical-dilution method (19).

Preparation of maxicells, protein labelling, and protein analysis. Maxicells of strain RYC1000 harboring plasmids were prepared as described previously (26). Labelling with 50 to 60 μCi of [35S]methionine was done for 15 min at 37°C with strong agitation. Labelled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (15). Gels were fixed, treated with 1 M sodium salicylate, and dried. For fluorography, gels were exposed to X-ray film (Konica AX) at −80°C.

RESULTS

Functional regions and directions of transcription in the MccH47 genetic system. The MccH47 genetic determinants, included in two adjacent HindIII restriction fragments, were subcloned into compatible high-copy-number plasmid vectors. The genetic system cloned into pUC13 was called pEX4 (16), and that cloned into pACYC184 was called pEX100. TnlacZ insertions mutagenesis of pEX100 was performed as described in Materials and Methods. Of 89 MudI1681 insertions, 38 (43%) bore deletions of 4 to 12 kb. This result probably occurred because of the size of the mutagenized plasmid (20.9 kb), which may be at the limit of the in vivo mutagenesis procedure with MudI1681. The mutant plasmids with deletions were discarded, and only those with the expected amount of DNA were studied further. For TnlacZ insertion mutagenesis of pEX100, 19% of the mutant plasmids gave rise to the Lac+ phenotype and, therefore, to translational fusions to lacZ. For both mutagenses, the insertion sites were physically located and correlated with the MccH47 phenotypes that they conferred on RYC1000 cells (Fig. 1). The directions of transcription of MccH47 genes were inferred from insertions that generated

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transcriptional (MudI1681) or translational (TnacZ) fusions to lacZ.

All pEX100::MudI1681 and pEX100::TnacZ mutant plasmids gave rise to clear-cut phenotypes, except for those containing insertions 3.2 and 49.0, which conferred partial phenotypes of MccH47 production and immunity. The external limits of the system are indicated by insertions 49.0 and 50.1, which resulted in wild-type MccH47 phenotypes. The results of the insertion mutagenesis experiments showed that the system spans 10.3 kb. Two DNA regions of ca. 1.1 and 5.5 kb are involved in MccH47 production. They are separated by a silent region of 3 kb in which insertions do not affect significantly MccH47 phenotypes and by a small segment involved in the immunity function. The pattern of genetic expression obtained is complex and reveals the existence of at least four different transcripts with alternating orientations (Fig. 1).

**Mapping of the immunity region.** Several subclonings in pUC13 or in vitro deletions in plasmids carrying the antibiotic system were performed. The derivative plasmids were physically analyzed, and the microcin phenotypes that they conferred were tested (Fig. 2). None of these plasmids conferred antibiotic production, but some of them conferred immunity to the cells. The immunity determinant, mchI, was thus localized in a minimal DNA fragment of 0.75 kb cloned into pUY10. The mchI gene(s) lies within this fragment and extends through the EcoRI and HindIII restriction sites contained in this cloned fragment.

**Complementation of the MccH47 production regions.** Complementation experiments to test the restoration of the production function were performed with plasmids derived from pEX4 and pEX100, with insertion mutations affecting the two regions of 1.1 and 5.5 kb involved in MccH47 production. Six complementation groups were determined and termed A, B, C, D, E, and F (Fig. 3).

No complementation between mutations located in the production region of 1.1 kb was obtained, thus defining complementation group A. The mutant plasmids used were those shown in Fig. 3. On the other hand, the mutant plasmid with insertion 7.2 complemented pEX4 derivative plasmids with Tn5 insertions in the 5.5-kb production region (28 and 73 in group C, 52 in group D, and 58 in group E) (Fig. 3).

Within the 5.5-kb production region five complementation groups could be identified.
Complementation group B was established by a unique Tn5 insertion mutation, 76, which was carried by a plasmid that complemented for production when coexisting in cells containing plasmids carrying mutations in groups C (7.7, 4.6, 3.1, 6.3, 4.7, and 7.3), D (3.5), and E (7.1) (Fig. 3).

Insertion mutations 7.7, 4.6, 3.1, 6.3, 4.7, and 7.3 did not complement mutations 28 and 18. Their complementation pattern with mutation 73 was particularly puzzling, because there was no complementation at all with mutation 7.3, while progressively larger halos of antibiosis appeared when 73 was tested with mutations 4.7, 6.3, 3.1, 4.6, and 7.7. Nevertheless, the diameter of these halos never exceeded one-third of that produced by cells carrying control plasmid pEX4 or pEX100. On the other hand, these pEX100 insertions were fully complemented for production in Tn5 mutations in groups B (76) and D (52 and B41). The same result was obtained with mutations 28, 18, and 73, which complemented mutation 7.2 in group A, mutation 3.5 in group D, and mutation 7.1 in group E. Complementation group C was therefore provisionally established as being limited by insertion 28 to the left and insertion 73 to the right (Fig. 3). For excluding possible negative interference, cells with pEX100 were transformed with pEX4::Tn5 mutation 73. The wild-type phenotype of MccH47 production conferred by pEX100 remained unaltered.

Insertion mutations 3.5, 52, and B41 constituted group D, since 3.5 did not complement 52 and B41. Insertions 52 and B41 were complemented by mutations in groups C (7.7, 6.3, and 7.3), E (30.5 and 7.1), and F (30.9), and insertion 3.5 was complemented by mutations in groups B (76) and C (28 and 73). The boundaries of group D were insertion 3.5 to the left and insertion B41 to the right. Just after B41 was a Tn5 insertion (84) that did not affect MccH47 phenotypes and hence revealed an intergenic region (Fig. 3).

Mutations 30.5, 30.4, 80.9, and 7.1 did not complement mutations 49, B58, and 26; thus, these mutations defined another complementation group, E, that was between two Tn5 insertions (84 and 46) that did not affect MccH47 phenotypes. Insertions 30.5, 30.4, 80.9, and 7.1 complemented mutations in groups C (18), D (52 and B41), and F (25 and 68); likewise, insertions 49, B58, and 26 complemented mutations in groups C (20.7) and F (90.4, 40.1, 80.4, and 30.9) (Fig. 3).

The sixth complementation group, F, was formed by insertion mutations 90.4, 40.1, 80.4, 30.9, and 10.4, which did not complement group F insertion mutations 25, B54, and 68. This group was limited by two insertions that did not affect MccH47 phenotypes (46 and 50.1). Mutation 80.4 complemented mutations in groups B (76), D (52), and E (49 and 26). Mutations 90.4, 40.1, and 30.9 complemented mutations B58 and 26 in group E. Likewise, insertions 25 and 68 complemented mutations 30.4 and 7.1 in group E (Fig. 3).

These results were further supported by complementation experiments with insertion mutations and subcloned restriction fragments from the MccH47 genetic system (Fig. 3). Plasmid pUY1, containing the left region devoted to micro-

**FIG. 3.** Complementation analysis of MccH47 production mutants. (i) Physical map of the cloned DNA in pEX4 or pEX100. The restriction sites are indicated as explained in the legend to Fig. 1. Only the EcoRI (E) sites used for subcloning experiments (see panel ii) are shown. The locations of insertions in pEX4 (lines above the map) and pEX100 (lines below the map) used in the complementation experiments are shown. Insertion mutations conferring wild-type phenotypes of production and immunity are represented by broken lines. Their identifying designations are given in parentheses. The complementation groups for MccH47 production, A, B, C, D, E, and F, are shown below the map. (ii) Subcloned restriction fragments used in the complementation experiments. pUY plasmids are pACYC184 derivatives, and pMVD plasmids are pACYC184 derivatives.
cin production, complemented insertion mutation 7.2 in group A. Likewise, insertion 76 (group B) was complemented by plasmid pMVD1, insertion 3.1 (group C) was complemented by plasmid pUY12, insertion 3.5 (group D) was complemented by plasmid pUY13, insertion 7.1 (group E) was complemented by plasmid pUY14, and insertions 90.4 and 10.4 (group F) were complemented by plasmid pUY15. Finally, plasmids pUY1 and pMVD2, each containing one of the DNA regions dedicated to MccH47 production—mchA, mchB, mchC, mchD, mchE, and mchF—and that correspond to the complementation groups described above.

Detection of MccH47 activity in nonproducing derivative clones. Nonproducing insertion mutants were lysed as described in Materials and Methods to detect the presence of microcin intracellularly. The clones analyzed harbored plasmids with insertion mutations affecting the microcin production genes mchA (7.2 and 5.6), mchB (76), mchC (7.7, 3.1, and 7.3), mchD (3.5), mchE (30.4), and mchF (90.4, 80.4, and 10.4) (Fig. 3). Intracellular MccH47 activity was found in mutants with insertions in the mchE and mchF genes. The antibiotic activity detected in extracts from cells bearing pEX100 or pEX4 was 12,800 U/ml; for clones harboring plasmids with insertions 30.4, 90.4, 80.4, and 10.4, this activity was about 6,400 U/ml, as quantitated by the critical-dilution method as described in Materials and Methods. The sensitive indicator strains used were BZB1011 and RYC10000 (pACYC184). All MccH47 preparations were assayed on a lawn of a control MccH47-immune strain, RYC10000 (pEX100), to determine the specificity of the antibiotic activity. These results demonstrated that mutants impaired in mchE or mchF expression can still synthesize active microcin H47 but fail at a subsequent step of antibiotic production, probably export to the extracellular medium.

TolC requirement for MccH47 production. The microcin B17 and colicin V genetic systems have been described to contain the determinants for dedicated expression of their respective antibiotics (14). An outer membrane protein, TolC, has been proposed to participate in some of these specific export mechanisms, e.g., those of hemolysin (28) and colicin V (11).

Strains MC4100 and MC4100 tolC::Tn5 were transformed with pEX4, and MccH47 production was assayed by the patch test. While MC4100 (pEX4) produced antibiotic halos 15 mm in diameter, MC4100 tolC::Tn5(pEX4) produced minimal halos 2 mm in diameter. The MccH47 production phenotype was recovered by transformation with pAX629 (12), a pACYC184 derivative carrying the tolC gene.

Silent region inside the system. There is a region of about 3 kb inside the system in which insertions did not significantly affect either MccH47 production or immunity. We therefore called it the silent region. Most of this region was deleted by taking advantage of Tn5 insertion 35 in pEX4, located 2.2 kb to the right of saII (see Fig. 5). As the only XhoI sites present in pEX4: Tn5 insertion 35 are those of the transposon, we performed a partial SaII digestion (because there is a SaII site in the pUC13 polynucleotid) and a total XhoI digestion, religated the fragments, and transformed RYC1000 cells. In this way, we generated a deletion of 2.2 kb inside the MccH47 genetic system which we called Δ35. The structure of Δ35 was confirmed by PstI digestions. This deletion, Δ35, devoid of most of the silent region, resulted in the same production phenotype as insertion 35, which resulted in halos only slightly smaller than those obtained with the control, pEX4. With regard to the immunity phenotype, clones bearing insertion 35 were as immune as those carrying pEX4, while clones bearing Δ35 showed a very low level of sensitivity. Nevertheless, the differences from the wild-type phenotypes were not significant, at least under the conditions used for testing.

Identification of MccH47 gene products. The gene products of pEX4, pEX100, their insertion derivatives, and subcolored fragments were analyzed in maxicell. Clones with several insertion mutations in pEX4 and in pEX100 within each complementation group were analyzed in this way, with identical results.

Figure 4A shows that a protein with an apparent molecular mass of 39 kDa corresponded to the gene product of mchA. The protein was found in cells with pEX4, pUY1, and mutant plasmid pEX4::Tn5 containing insertion 19, which lies inside the silent region of the system. However, it was absent from cells with pUY2, a subclone that does not comprise the entire mchA gene, and from cells with mutant plasmids containing insertions B4, B53, and 21, which are located at the left and right limits of complementation group A (see Fig. 5).

Figure 4B shows that there was an apparent 43.5-kDa protein corresponding to a gene, called mchS1, located in the silent region of the system. This protein was present in cells bearing pEX4, pUY6, and a plasmid containing insertion 38 and absent from cells bearing pUY8 (in which a fusion product was generated) or mutant plasmids containing insertions 19 and 51 (see Fig. 5).

In Fig. 4C, an apparent 54-kDa protein was identified as the gene product of mchC. As this protein comigrated with the inhibitor of the IS50 transposase, XhoI deletions were made in four pEX100::TnlacZ mutant plasmids (containing insertions 7.3, 3.1, 4.6, and 7.7), affecting mchC, and in a control plasmid (containing insertion 7.8), in which the TnlacZ insertion lies outside the MccH47 genetic system (see Fig. 5). As the cloned DNA in pEX100 does not contain XhoI sites, an XhoI deletion in a pEX100::TnlacZ derivative only removes from TnlacZ a 2,455-bp fragment that includes most of the IS50R (3). Therefore, these XhoI deletion derivative plasmids (denoted with an asterisk) will not code for either the transposase (58 kDa) or the inhibitor of the transposase (54 kDa) but will still code for kanamycin resistance (25 kDa). The 54-kDa protein was present in cells carrying pEX100 and 7.8* but was absent from those bearing 7.3*, 3.1*, 4.6*, and 7.7*.

Figure 4D shows that two proteins with apparent molecular masses of 30.5 and 45 kDa were gene products of mchE. These two proteins were present in cells with pEX4, pUY2, and pEX100 but absent from cells with plasmids containing insertions 26, B58, and 7.1.

Figure 5 shows the overall organization of the MccH47 genetic system, summarizing the results obtained in this work. The locations of insertions in all the mutant plasmids used in maxicell experiments are shown, as are the identified gene products. Cistrons and directions of transcription identified in the system are also shown.

DISCUSSION

The MccH47 genetic determinants were previously cloned from the chromosome of a wild-type E. coli strain. Transposon mutagenesis showed that they are located on a continuous DNA segment of about 10 kb (16). The work
described here presents a general characterization of the MccH47 genetic system.

Further transposon mutagenesis and functional analyses of subcloned fragments revealed that two DNA regions of 1.1 and 6.3 kb, physically connected by a 3-kb DNA stretch, are required for the expression of the antibiotic functions. The immunity determinant is located on an 0.8-kb DNA fragment. Complementation analysis of mutants impaired in the production function showed that the system contains at least six production genes. The 1.1-kb region is devoted to microcin production and contains one production gene, mchA. The 6.3-kb region contains the immunity determinant, mchI, and five production genes. The transcriptional organization of the system appears to be complex, comprising at least four transcripts with alternating directions.

On the basis of the results of the previously reported Tn5 mutagenesis analysis, it was not possible to locate the immunity determinant. Clones bearing the Tn5 mutant plasmids, all derivatives of strain BZB1011, were almost all impaired, to some extent, in the immunity function (16).

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**FIG. 4.** Identification of mchA, mchS1, mchC, and mchE gene products. SDS-PAGE was used to analyze $^{35}$S-labelled proteins from maxicells harboring the indicated plasmids (see also Fig. 2 for the description of pUY plasmids and Fig. 5 for the insertion mutations used). Asterisks designate XhoI deletions in pEX100::TnlacZ derivatives used to identify mchC (C). Maxicells were prepared as described in Materials and Methods, and plasmid-encoded proteins were labelled and analyzed by 15% SDS-PAGE. Size standards are indicated in kilodaltons. The arrows indicate the positions of the gene products corresponding to mchA (A), mchS1 (B), mchC (C), and mchE (D).

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**FIG. 5.** Cistrons and gene products of the MccH47 genetic system. (a) Physical map of the cloned DNA in pEX4 or pEX100 and locations of insertion mutations (pEX4 derivatives above the line and pEX100 derivatives below the line) used for the identification of gene products. The extension of the MccH47 genetic system is shown above. (b) Sizes in kilodaltons of the proteins identified as gene products of mchA, mchS1, mchC, and mchE. (c) Directions of transcription inside the system. (d) Identified MccH47 genes (mch). mchA, mchB, mchC, mchD, mchE, and mchF are genes involved in antibiotic production, mchS1 is a gene from the silent region, and mchI is the immunity determinant.
Later on, Tn5 mutant plasmids were introduced into RYCl000 cells. The analysis of the immunity phenotype of the resulting clones gave rise to results strictly coincident with those obtained with the pEX100 mutants used in this work. Only two mutant plasmids conferred partial phenotypes of production and immunity, and their insertions mapped to the DNA region in which the immunity determinant has been located.

The search for MccH47 activity inside the nonproducing mutant cells revealed that four production genes were devoted to microcin biosynthesis—mchA, mchB, mchC, mchD—and that the remaining two were dedicated to antibiotic export to the extracellular medium—mchE and mchF. The immunity gene, mchl, and genes mchB, mchC, and mchD are contiguous and are transcribed in the same direction. This fact opens the possibility of their being arranged as an operon. Moreover, the partial antibiotic phenotypes observed for mutant insertions in the immunity region may be due to polar effects of their mutations on the expression of these four genes. Nevertheless, data from the complementation experiments indicated that mchC and mchD are able to be expressed independently. The abundance of MccH47 genes involved in antibiotic biosynthesis is reminiscent of the microcin B17 and C7 systems, which contain a structural gene for a precursor of the microcin and several other genes devoted to introducing posttranslational modifications of this primary product (14, 22).

MccH47 export depends on the mchE and mchF gene products. These genes are transcribed in the same direction and, most probably, from independent promoters, since a Tn5 insertion between them did not affect the microcin phenotypes. The microcin B17 and colicin V systems also contain a set of two linked genes dedicated to the export of their antibiotics, which are exported by a signal sequence-independent pathway. The parallelism between the MccH47 and ColV systems is particularly noteworthy in this regard. ColV secretion requires the products of genes cvaA and cvaB of its antibiotic system and the chromosomally encoded protein TolC (11). Mutations in cvaA abolish the production of two proteins of ca. 43 and 27 kDa, resulting from two different in-frame translation initiation sites (10, 11). The mchE gene also codes for two proteins with apparent masses of 45 and 30.5 kDa. Pulse-chase experiments (data not shown) indicated that these two products do not result from protein processing. The DNA sequence of the cvaB gene predicts a protein of at least 78 kDa that has not yet been detected (10). The size of MccH47 gene mchF also predicts a protein of at least 64 kDa that could not be detected in maxicells. An additional resemblance is the involvement of minor outer membrane protein TolC in MccH47 production as well as in ColV secretion. All these similarities suggest that MccH47 may share with ColV a similar mechanism of antibiotic export (11, 14).

Transposon insertions into the 3-kb DNA region between mchA and mchl had a minimal or null effect on microcin functions. This region was thus qualified as “silent,” but this conclusion does not mean that it is not expressed. In fact, this region codes for the MchS1 polypeptide. In addition, lac fusions to the region resulted in high levels of β-galactosidase activity.

In summary, the MccH47 genetic system exhibits significant similarities to those of other described microcins. However, none of these previously studied microcin genetic systems have a chromosomal location, nor do they include a silent region, as was found for MccH47. At present, we do not know whether this region is functionally related to the MccH47 antibiotic phenomenon, but if it proves to be so, then this genetic system is certainly more extensive and complex than those of other known microcins.

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**REFERENCES**