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Genetic Analysis of Plasmid Determinants for Microcin J25 Production and Immunity

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Microcin J25 (MccJ25) is a small peptide antibiotic produced by an *Escherichia coli* strain isolated from human feces. The genetic determinants for MccJ25 synthesis and immunity have been cloned from the low-copy-number wild-type plasmid pTUC100 into the compatible vectors pBR322 and pACYC184. Physical and phenotypical analysis of insertion mutations and complementation tests defined three contiguous genes involved in MccJ25 production which span a region of about 2.2 kb. Immunity to the antibiotic is provided by an additional gene adjacent to the production region.

Microcins are small peptide antibiotics produced by members of the family *Enterobacteriaceae* (1). They have been classified into six groups (A, B, C, D, E, and H) according to cross-immunity criteria (2, 7). We have described a novel microcin produced by *Escherichia coli* AY25, isolated from human feces (10). Although in previous reports it was designated microcin 25 (Mcc25), it seems more appropriate to include it in a new immunity group (J); therefore, from now on we shall call it microcin J25 (MccJ25).

As with other microcins, production of MccJ25 is plasmid determined and takes place during the stationary phase of growth (10). Iron limitation increases MccJ25 production (12).

The multifunctional outer membrane protein FhuA serves as a receptor for MccJ25, which is then internalized through the TonB pathway and the SbmA protein (11, 13). The SbmA protein is also required for the penetration of microcin B17 (8).

We report here the cloning and analysis of the MccJ25 genetic system, which show that at least three genes are involved in MccJ25 synthesis and that an additional gene is responsible for immunity to the antibiotic.

Microcin production was detected by stabbing single colonies onto Luria-Bertani plates overlaid with a hypersensitive strain of *Salmonella newport* (10). MccJ25 immunity was determined by a cross-streaking assay as described previously (10). Plasmid DNA preparation, digestions, ligations, transformations, and electrophoresis were done by standard methods (14). Tn*5* insertion mutagenesis was performed essentially as described previously (5).

Cloning of the MccJ25 genetic system. To identify regions of the wild-type plasmid pTUC100 (10) encoding MccJ25 functions, we carried out two shotgun cloning experiments by using the endonucleases *Pst*I and *Bam*HI in one experiment each and the vector pBR325 in both. The ligation mixtures were used to transform *E. coli* MC4100. Chloramphenicol-resistant transformants were selected and screened for microcin production. While the cloning experiment using *Pst*I was unsuccessful, several of the clones containing *Bam*HI fragments produced microcin and were immune to it. For each of these clones tested, the pBR325 derivative contained a 13-kb *Bam*HI fragment insertion. The fragment from one of these recombinant plasmids, named pTUC101, was subcloned in pA CYC184, resulting in the plasmid pTUC201 (Fig. 1). Sequential *Sal*I and *Hin*dIII deletions were then made, yielding plasmids pTUC202 and pTUC203, respectively (Fig. 1). The latter still retained both microcin production and immunity and was composed of a 5.2-kb fragment and the vector (3.6 kb). Halos of antibiosis generated by cells harboring any of these recombinant plasmids were notably larger than those observed with the wild-type, low-copy-number pTUC100. This effect is likely due to the increased dose of microcin genes.

Tn*5* mutagenesis and complementation analysis. To define the microcin production region and to conduct complementation experiments, we constructed pTUC341, a derivative of pBR322 which contains the same *Hin*dIII-*Sal*I fragment as pTUC203 and is compatible with the latter. Plasmids pTUC 203 and pTUC341 were subjected to Tn*5* mutagenesis. All pTUC203::Tn*5* and pTUC341::Tn*5* mutant plasmids gave rise to clear-cut phenotypes: colonies which produced normal amounts of microcin and colonies which did not produce microcin at all. No transposon mutants which had lost immunity to MccJ25 were found, probably because microcin-producing cells are inviable in the absence of immunity. Independent Tn*5* insertions in each plasmid which eliminated microcin production were mapped by restriction enzyme digestions (Fig. 2). Complementation analysis using all possible combinations of these two sets of mutant plasmids showed that there were three distinct complementation groups which spanned a region of about 2.2 kb. Insertion mutations pTUC203-19, -6, -8, -12, -2, -15, -7, and -4 and pTUC341-7 did not complement each other, thus defining complementation group A, but they were complemented by all other Tn*5* mutations. Likewise, pTUC203-13, -1, -10, -9, -18, -14, and -3 and pTUC341-11, -3, -16, -9, and -14 formed group B, and group C comprised mutations pTUC 203-16 and -20 and pTUC341-5, -13, -8, -4, -6, and -15. The three genes corresponding to these complementation groups were designated *mcjA*, *mcjB*, and *mcjC* (Fig. 2). The approximate gene sizes are estimated as 80 to 280 bp for *mcjA*, 400 to 700 bp for *mcjB*, and 400 to 1,100 bp for *mcjC*.

It should be noted that in contrast to the full complementation observed with mutations in groups A and B, we found

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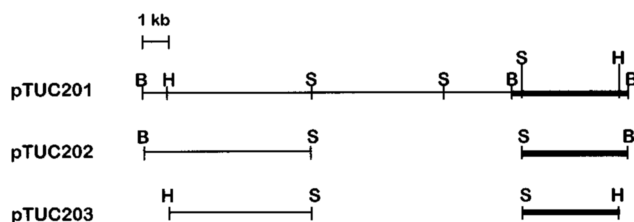

FIG. 1. Structure of plasmid pTUC201 and its derivatives. pTUC201 was constructed by cloning a 13-kb fragment (thin line) containing the MccJ25 genetic system into the vector pACYC184 (heavy line). The plasmid is shown linearized at one of the insert-vector junction *Bam*HI sites. Plasmids pTUC202 and pTUC203 were made from pTUC201 by single restriction enzyme digests followed by religation. Restriction sites: B, *Bam*HI; H, *Hin*dIII; S, *Sal*I.

partial complementation with mutations of groups B and C. In fact, this combination of plasmid mutants produced small amounts of antibiotic. A possible explanation is that *mcjB* and *mcjC* are part of the same operon and that the transposon exerts a polar effect on the downstream gene. The low level of microcin production observed in the complementation tests can be attributed to an incomplete polarity of the Tn*5* insertions. Although Tn*5* is known to be strictly polar in most cases, insertions that result in constitutive, low-level expression of distal genes have been observed previously (3), and this effect is observed more readily when Tn*5*-induced insertion mutations in operons that are carried by multicopy plasmids are analyzed.

The results of complementation experiments with restriction fragments from the system subcloned separately into pUC18 were in agreement with those of the Tn*5* mutational analysis. The plasmid containing the 700-bp *Hin*dIII-*Eco*RI(1) fragment from the left region of microcin production complemented the insertion mutations of group A but not those of group B. When the fragment was in the opposite direction, in pUC19, the same results were observed. This indicated that only the *mcjA* gene is carried intact on this fragment and that it has its own transcriptional promoter. When subcloned, the 0.3-kb *Eco*RI(1)-*Eco*RI(2) fragment was unable to complement the mutants of group B, but they were complemented by the 1.5-kb *Hin*dIII-*Eco*RV(1) fragment. These results, together with the Tn*5* insertion data, demonstrated that the *mcjB* gene starts to the left of the *Eco*RI(1) site and ends at the right of the *Eco*RI(2) site, before the *Eco*RV(1) site. On the other hand, the *Eco*RI(2)-*Eco*RI(3) fragment did not complement mutations in group C when subcloned in either orientation, suggesting that the *mcjC* gene could have been truncated by the *Eco*RI digestion. Since the left-hand boundary of *mcjC* must lie to the right of the *Eco*RI(2) site, this gene would extend beyond the *Eco*RI(3) site. It is also possible that the entire gene is contained in the fragment but is not properly transcribed or that efficient translation of *mcjC* is coupled to the translation of *mcjB*.

Identification of the MccJ25 immunity determinant. To localize the sequences required for MccJ25 immunity, we digested pTUC101 with *Eco*RI, and the fragments were ligated with DNA of plasmid pBR322 cleaved at this site. The ligation mixture was used to transform DH5α-sensitive cells. Several recombinant clones conferred complete immunity and, as expected, none of these constructs produced any detectable microcin. They contained a recombinant plasmid, designated pJS100, with an inserted fragment of 2.2 kb. This fragment was then recloned into the *Eco*RI site of another vector, pUC18, giving rise to plasmid pJS300. After a restriction map of the fragment was generated (Fig. 3), deletion derivatives of the fragment were obtained by directional deletion from the unique sites of the pUC18 polylinker. Figure 3 shows the extent of deletions which resulted in loss of immunity. These results indicated that the left-hand boundary of the immunity locus must lie between the *Eco*RI and *Acc*I sites and the right-hand boundary must lie between the *Hin*cII and *Eco*RI sites. To define further the limits of the immunity determinant(s), plasmid pJS300 was mutagenized with Tn*5*, and MccJ25 immunity-deficient derivatives were analyzed for mapping the transposon. Figure 3 shows the locations of 13 independent Tn*5* insertions which abolished the immunity phenotype. To determine the number of cistrons within the immunity region, we did a complementation analysis. The 2.2-kb frag-

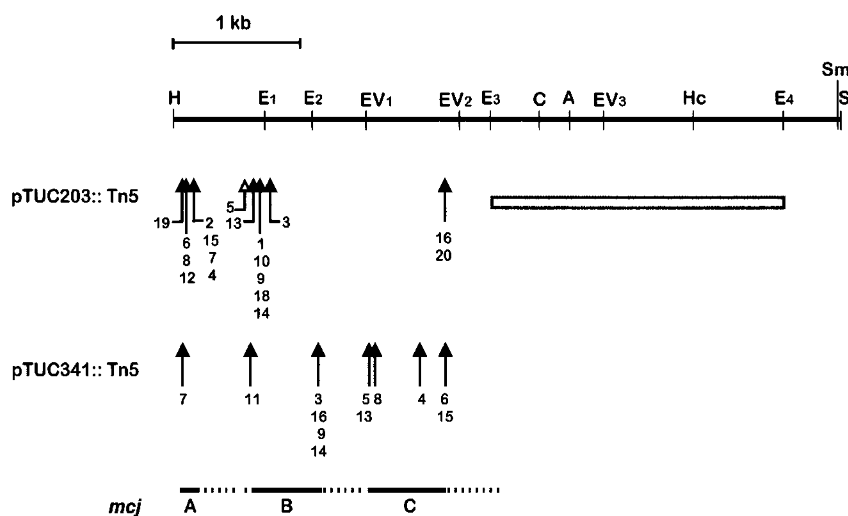

FIG. 2. Restriction endonuclease map of the *Hin*dIII-*Sal*I (5.2-kb) fragment cloned into pACYC184 and pBR322 to generate plasmids pTUC203 and pTUC341, respectively. The fragment did not have sites for *Pst*I, *Bgl*II, *Bam*HI, *Sac*I, and *Kpn*I. The positions of the various Tn*5* insertions in each plasmid that resulted in inactivation of MccJ25 production are indicated by filled arrows. The open arrow (insertion pTUC203-5) corresponds to an insertion which did not affect the MccJ25 phenotype, thus defining an intergenic region. The minimum lengths of the three *mcj* complementation groups, *mcjA*, *mcjB*, and *mcjC*, are represented by solid lines, and the dotted lines indicate the maximum possible distances covered by the regions. The horizontal open bar indicates the region which, when cloned, confers MccJ25 immunity on host cells. Restriction sites: H, *Hin*dIII; E, *Eco*RI; EV, *Eco*RV; A, *Acc*I; C, *Cla*I; Hc, *Hin*cII; Sm, *Sma*I; S, *Sal*I.

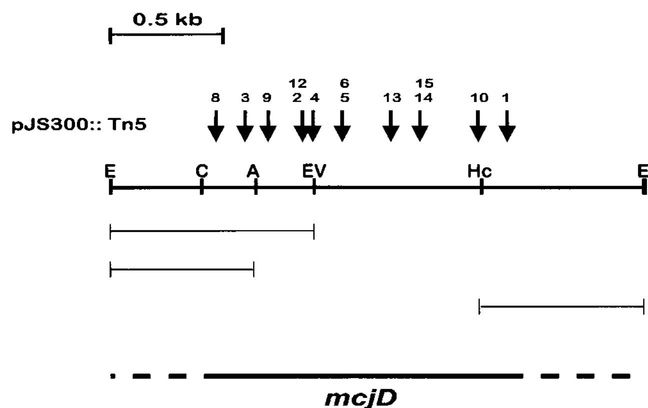

FIG. 3. Restriction endonuclease map of the immunity region cloned into pUC18 to generate pJS300. The arrows indicate sites of Tn*5* insertions in the fragment which gave an Imm$^-$ phenotype. The thin lines below the map represent the extent of deletions into the fragment which resulted in loss of immunity. The thick line at the bottom represents the minimum length of the immunity gene *mcjD*, and the dashed lines indicate the maximum possible length. Restriction sites are as defined in the legend to Fig. 2.

ment containing the immunity region was subcloned in pA CYC184, and the hybrid plasmid, termed pJS200, was mutagenized with Tn*5*. Complementation experiments with all possible combinations of pJS300::Tn*5* and pJS200::Tn*5* Imm$^-$ derivatives revealed the existence of one complementation group, which defined a gene designated *mcjD*, whose approximate size is estimated as 1,200 to 2,200 bp (Fig. 3).

MccJ25 activity in nonproducing insertion mutants. To determine whether *mcjA*, *mcjB*, and *mcjC* are involved in the export of MccJ25, pTUC203::Tn*5* insertion mutants affected in these genes, as well as control cells harboring pTUC203, were lysed as described previously (4) to detect the presence of intracellular microcin. Doubling dilutions of the lysate supernatants were spotted onto a lawn of sensitive cells. The microcin titer was the reciprocal of the last dilution giving a clear inhibition zone. Microcin activity was also quantitated in culture supernatants. As shown in Table 1, no microcin was detectable in culture supernatants or inside cells of *mcjA*, *mcjB*, and *mcjC* mutants. Since export mutants would likely accumulate microcin intracellularly, none of these genes appears to be responsible for release of the antibiotic to the extracellular medium.

Concluding remarks. We have shown that the MccJ25 biosynthetic region consists of at least three genes, *mcjA*, *mcjB*, and *mcjC*, which are close to each other, suggesting a polycistronic operon structure. Given the low molecular weight of MccJ25 (~2,100) (10), we consider *mcjA* a strong candidate for being the structural gene because of its small size. The immunity gene, *mcjD*, resides within a 2.2-kb region adjacent to the *mcjC* gene (Fig. 2). Likewise, production and immunity genes are closely linked in the cases of microcins B17, C7, and H47 (4, 9, 15, 16), colicin V (5), and many ribosomally synthesized peptide antibiotics from gram-positive bacteria (6). Production regions for these antibiotics usually include export-related genes. Although this seems not to be the case for MccJ25, the possibility that the immunity gene, *mcjD*, may play a role in export should be considered.

TABLE 1. Microcin J25 activity in culture supernatants and lysed cells

Plasmid	Genotype	MccJ25 titer[a] in:	
		Lysed cells	Supernatant
pTUC203	*mcj*$^+$	32	64
pTUC203-6	*mcjA*	<1	<1
pTUC203-1	*mcjB*	<1	<1
pTUC203-16	*mcjC*	<1	<1

[a] Determined by the critical dilution method, as described in the text. <1, below the detection limit of the method.

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