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appR Gene Product Activates Transcription of Microcin C7 Plasmid Genes

LOLA DÍAZ-GUERRA, FELIPE MORENO, AND JOSÉ L. SAN MILLÁN*

Unidad de Genética Molecular, Servicio de Microbiología, Hospital Ramón y Cajal, Madrid 28034, Spain

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Microcin C7 (MccC7) is encoded by *Escherichia coli* plasmid pMccC7. However, some strains of *E. coli* K-12 carrying this plasmid do not produce this antibiotic. Here we show that these strains differ in the gene locus *appR*. This chromosomal gene product controls MccC7 production by activating the transcription of some, but not all, MccC7 plasmid genes.

Microcin C7 (MccC7) is a 1,000-dalton peptide antibiotic active against enterobacteria (3). It inhibits protein synthesis in *Escherichia coli* cells (4). MccC7 is produced by *E. coli* strains carrying pMccC7, a 43-kilobase conjugative IncX plasmid (9). By introducing this plasmid into several *E. coli* K-12 strains from our laboratory collection, we found that such strains can be grouped into two classes: those that produced inhibition halos 1 cm in diameter around the inoculum (MC4100 and its derivatives) and those that produced halos <0.4 cm in diameter or no halo at all (C600, AB1157, and BZB1011). These results indicate that some wild-type strains could carry a chromosomal mutation that interferes with MccC7 production.

Plasmid genes responsible for MccC7 production have been mapped to a DNA segment of about 5.0 kilobases of pMccC7. This segment could be divided into four regions (α, β, τ, and δ) on the basis of the phenotypic features of insertion mutations in each of them (9). The nonadjacent regions β and δ are also involved in MccC7 immunity (9). In-frame *lacZ* gene fusions in three of these regions, α, τ, and δ, were also obtained. More recently, we have obtained *lacZ* operon fusions in regions α, β, and δ by using mini-Mu dI1681 (L. Díaz-Guerra, unpublished data). All of the regions are transcribed in the same direction. To get some insight into the cause of the lack of production in some *E. coli* K-12 strains, we introduced multicopy plasmids carrying these *mcc-lacZ* fusions in both strains pop3351 (Mcc^+ Lac^-) and CID128 (Mcc^- Lac^-; derivative of BZB1011) (Table 1) and compared their β-galactosidase activities. Expression of fusions to regions α and β was lower in CID128 cells, 10-fold in the case of protein fusions to region α and 3- to 5-fold in the case of operon fusions to α and β (Table 2). A similar difference between these strains was found when they were transformed with monocopy plasmid pDG250, thereby eliminating any possible artifact due to the high copy number of the plasmids (plasmid pDG250 is described in the legend to Fig. 1). The levels of expression of gene and/or operon fusions in regions τ and δ were the same in both strains. These results seemed to indicate that such differences between the two classes of strains could result from a chromosomal mutation which reduces the transcription of one or more of the plasmid genes required for MccC7 production.

To map the putative chromosomal gene involved in MccC7 production, we took advantage of the different β-galactosidase activities shown by producer and nonproducer strains harboring plasmid pDG250. Indeed, while strain CID128(pDG250) could hardly grow in lactose medium supplemented with phenylethyl-β-D-thiogalactoside (10^{-3} M), a competitive inhibitor of β-galactosidase, strain pop3351(pDG250) grew fairly well. Strain CID128(pDG250) was transduced with a P1 lysate grown on a pool of random Tn*10* insertions in pop3351. Tetracycline-resistant transductants were selected on minimal-lactose plates containing phenylethyl-β-D-thiogalactoside. Three clones which gave large colonies were retained for further study. Linkage between the Tn*10* insertions and the Lac^+ phenotype was confirmed by P1 transduction. When plasmid pMM402, a derivative of pMccC7 carrying Tn*3*, was introduced into these clones by conjugation, they produced as much MccC7 as did strain pop3351. This indicated that production of MccC7 was correlated with activation of the α plasmid determinant by a chromosomal gene product.

Preliminary Hfr mapping experiments indicated that a Tn*10* insertion which was 35% cotransducible with the Lac^+ Mcc^+ phenotype was located between 55 and 61 min on the *E. coli* chromosome map (data not shown). P1-mediated transduction experiments were performed to map the mutation more precisely. A P1 phage stock was prepared on strain BZB1011 and used to transduce strain GY3442 (pMM402), a normal producer of MccC7, to Cys^+ or Arg^+. The Mcc^- phenotype of BZB1011 was found to be cotransducible with *cysC* (33%) and *argA* (2%). These results suggested that the locus involved in MccC7 production is located near the *cysC* gene, which maps at 59.5 min of the *E. coli* chromosomal map, between *appR* (59 min) and *relA* (60 min) (12). Since strain pop3351 is $RelA^-$ and strain BZB1011 is $RelA^+$, we checked the possibility that gene *relA* was involved in MccC7 production. However, this seems not to be the case, because different isogenic $relA^+$ and $relA^-$ strains apparently produced identical amounts of MccC7. Moreover, Tc^r Mcc^+ transductants of strain BZB1011 were able to grow on medium M63-AT plates (11), on which *relA* mutants do not grow because of their sensitivity to 3-amino-1,2,4-triazole.

To determine whether the mutation affecting MccC7 production mapped at the *appR* locus, we assayed the level of acid phosphatase produced by strain BZB1011 and its Mcc^+ derivatives. This enzyme is encoded by gene *appA*, whose expression is regulated at the level of transcription by the product of the *appR* locus; *appR* mutants produce three to four times less acid phosphatase than do their $appR^+$ isogenic strains (12). Surprisingly, while strain BZB1011 produced 18 U of acid phosphatase, the Mcc^+ derivatives produced 53 U, suggesting that the Mcc^- phenotype in strain

* Corresponding author.

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

Strain	Genotype	Source or reference
MC4100	F⁻ *araD139 ΔlacU169 rpsL relA thiA*	2
pop3351	MC4100 *ΔmalB1*	5
BZB1011	F⁻ *gyrA*	10
CID128	BZB1011 *ΔlacU169*	This work
GY3442	F⁻ *thi-1 lysA22 thyA61 argA21 relA1 cysC43 pheA97 spc-339 malA1 xyl-7 mtl-2 lam*	P. Boquet
CP78	F⁻ *thr-1 leuB6 his-65 argH46 thi-1 ara-13 gal-3 malA1 xyl-7 mtl-2 tonA2 supE44*	B. J. Bachmann
CP79	CP78 *relA2*	B. J. Bachmann
SBS911	F⁻ *Δgal-165 lacL8 srl*::Tn*10*	P. Boquet
SBS912	SBS911 $appR_{XE8}$	P. Boquet
SBS915	SBS916 $appR_{XE9}$	P. Boquet
SBS916	F⁻ *Δgal-165 lacL29 srl*::Tn*10*	P. Boquet

[a] We also used different Hfr strains from our laboratory collection, including KL14, KL16, KL983, pop3000, etc.

BZB1011 was indeed due to a mutation in *appR* (acid phosphatase assays were done as described by Touati et al. [12]). Plasmid pMM501, a pMccC7 derivative carrying Tn*5*, was then introduced into isogenic strains carrying two different *appR* alleles. In all cases, wild-type strains (SBS911 and SBS916) produced normal amounts of MccC7, while the corresponding *appR* strains (SBS912 and SBS915) did not produce the antibiotic. Consequently, we concluded that the *appR* gene product is involved in MccC7 production.

This conclusion was strengthened by isolation of mutants impaired in MccC7 production. Strain pop3351, harboring plasmid pMM501, was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Adelberg et al. (1). More than 5,000 colonies were tested for MccC7 production. In most cases, the mutations responsible for the Mcc⁻ phenotype were located in the plasmid. However, in four cases they were chromosomal, as indicated by the fact that transfer of resident plasmid pMM501 to plasmid-free *appR*⁺ cells yielded normal MccC7 producers. These four mutants were then assayed for acid phosphatase activity, and three of them (721, 731, and 742) showed reduced activity compared with that of parental strain pop3351. The level of the enzyme measured in these mutants fell within the characteristic range of *appR* mutants, indicating that these mutations impair both MccC7 production and acid phosphatase synthesis. This hypothesis was confirmed by transducing the

TABLE 2. Expression of *lacZ* fusions to different DNA regions involved in MccC7 production in *E. coli* strain pop3351 or CID128

DNA region[a]	β-Galactosidase U[b]	
	pop3351	CID128
Gene fusions		
α	4,150	400
τ	900	800
δ	100	100
Operon fusions		
α	1,690	285
β	145	50
δ	90	85

[a] Fusions were carried by multicopy plasmids derived from pDG104 (9).
[b] Assayed when cultures in LB medium reached the stationary phase (expressed in Miller units [8]).

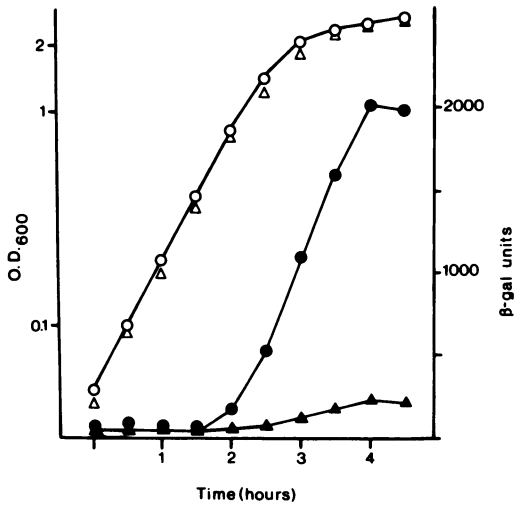

FIG. 1. Time course of β-galactosidase induction from strains SBS915 *appR*(pDG250) (△) and SBS916(pDG250) (○). The open symbols indicate optical densities at 600 nm, and the closed symbols indicate β-galactosidase units. pDG250 is a monocopy plasmid carrying a *lacZ*-gene fusion in region α. It was constructed by cloning a *Hin*dIII fragment from multiple-copy fusion plasmid p3.6 (9) into pMM6165, a pMccB17 miniplasmid described elsewhere (7). The fragment includes pMccC7 DNA upstream from the MccC7 region, the hybrid gene, and the kanamycin resistance determinant from mini-Mu dII1681. The bacteria were grown aerobically at 37°C in LB medium.

Tn*10*-linked *appR*⁺ gene into mutant 742. Of the tetracycline-resistant transductants, 25% were Mcc⁺, and only these transductants had the normal level of acid phosphatase activity found in *appR*⁺ strains. Other transduction experiments showed that the mutation conferring the Mcc⁻ phenotype mapped near *cysC*, as expected if it was located in the *appR* locus (data not shown).

All of the results presented above indicated that AppR activates the transcription of the adjacent α and β MccC7 plasmid regions. Synthesis of MccC7 is induced when cultures cease exponential growth (3), and studies on transcriptional regulation using plasmid pDG250 indicated that production of the hybrid protein was induced when the cells ceased exponential growth. β-Galactosidase activity increased at least 100-fold in producing strain pop3351 (L. Díaz-Guerra, unpublished data). To further study the effect of the *appR* gene product on the transcription of MccC7 genes, plasmid pDG250 was introduced into strains SBS915 (*appR*) and SBS916 (*appR*⁺) and their β-galactosidase activities were determined at different times through exponential and stationary growth. Both strains produced very little β-galactosidase activity (10 to 20 U) during logarithmic growth (Fig. 1). However, as the *appR*⁺ cells entered the stationary phase, β-galactosidase activity suddenly increased to levels at least 100-fold higher. Although an increase of activity was also observed in cultures of *appR* cells entering the stationary phase, it was only 10-fold higher. These results are consistent with both the appearance of MccC7 activity in the stationary phase and with the Mcc⁻ phenotype of *appR* mutants, and we concluded that these mutants interfere with MccC7 production by reducing the stationary-phase activation of one or more of the MccC7 genes.

The product of the *appR* gene seems to play a pleiotropic role, since *appR* mutants are unable to start growth normally

under anaerobic conditions (12). It has been proposed that the gene codes for a positive effector essential for growth under anaerobic conditions (12). Moreover, the *appR* gene product activates expression of gene locus *appA*, which codes for pH 2.5 acid phosphatase, at a transcriptional level (12). In fact, the regulation of MccC7 production resembles that of acid phosphatase in that the synthesis of both is induced only when a culture enters the stationary phase. However, the fact that *appR* strains also slightly increased expression of *mcc-lacZ* fusion in the stationary phase (Fig. 1) indicates that the two types of control are independent.

Regulation of MccC7 production resembles that of another peptide antibiotic, microcin B17, in that both are induced in the stationary phase. Moreover, although *appR* does not control microcin B17 expression, its synthesis is regulated by another chromosomal regulatory product, protein OmpR (6), which does not affect MccC7 production (5). It is interesting that two genetic systems that exhibit a number of similarities have evolved by using different transcription activators. The study of the *appR*- and stationary-phase-regulated MccC7 promoter will allow us to compare the two systems and to examine gene expression in resting *E. coli* cells. Moreover, it would also be interesting to know whether other conditions affecting acid phosphatase synthesis, such as anoxia, limitation of PP_i, catabolite repression, or inhibition by cyclic AMP (12), also regulate MccC7 production.

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