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DNA Sequence, Products, and Transcriptional Pattern of the Genes Involved in Production of the DNA Replication Inhibitor Microcin B17

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The 3.8-kilobase segment of plasmid DNA that contains the genes required for production of the DNA replication inhibitor microcin B17 was sequenced. The sequence contains four open reading frames which were shown to be translated in vivo by the construction of fusions to *lacZ*. The location of these open reading frames fits well with the location of the four microcin B17 production genes, *mcbABCD*, identified previously through genetic complementation. The products of the four genes have been identified, and the observed molecular weights of the proteins agree with those predicted from the nucleotide sequence. The transcription of these genes was studied by using fusions to *lacZ* and physical mapping of mRNA start sites. Three promoters were identified in this region. The major promoter for all the genes is a growth phase-regulated OmpR-dependent promoter located upstream of *mcbA*. A second promoter is located within *mcbC* and is responsible for a low-level basal expression of *mcbD*. A third promoter, located within *mcbD*, promotes transcription in the reverse direction starting within *mcbD* and extending through *mcbC*. The resulting mRNA appears to be an untranslated antisense transcript that could play a regulatory role in the expression of these genes.

The microcins constitute a family of low-molecular-weight polypeptide antibiotics produced by many different species of enteric bacteria (1, 3). The spectrum of action of most microcins also includes many different members of the family *Enterobacteriaceae*. Microcin B17 (MccB17) is the prototype of the group B microcins (1, 3). MccB17 has been shown to be a peptide of about 3,200 daltons of bactericidal action which inhibits the elongation process of DNA replication (10, 17). Like most other microcins, the production of MccB17 is plasmid determined. The wild-type plasmid producing MccB17 is a 70-kilobase-pair *incF2* plasmid called pMccB17 (2, 28). A 6.3-kilobase-pair *Bam*HI-to*Bgl*II fragment of DNA from pMccB17 codes for the production of MccB17 as well as for the immunity to this antibiotic (13, 28). Genetic complementation studies showed that four genes, designated *mcbABCD*, were necessary for production (29), while three additional genes, *mcbEFG*, confer immunity on the producing strain (12). The production genes were located in a 3.8-kilobase-pair region spanning from the *Bam*HI site to a *Sal*I site.

Through the analysis of transcriptional fusions constructed with Mu d1(Ap *lac*), we showed that transcription proceeds from the *Bam*HI site towards the *Sal*I site (16). Those studies also showed that there is a promoter located somewhere between the *Bam*HI site and the beginning of *mcbA*. Expression of the transcriptional fusions is regulated by the product of the *ompR* gene. For fusions in *mcbABC*, there is also temporal regulation of expression. The maximum levels of transcription are observed during stationary phase. The promoter responsible for this behavior is located 60 bases upstream of *mcbA* (8).

To understand the production of MccB17 and its regulation, we have determined the DNA sequence of the production genes, identified the gene products and their corresponding coding regions, and studied the transcriptional pattern of these genes.

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MATERIALS AND METHODS

Strains, plasmids, DNA manipulations, maxicells, and assays. Strains and plasmids used in this study are listed in Table 1. Plasmid pSL100 contains a promoterless chloramphenicol resistance gene flanked by transcriptional terminators to prevent its expression. Only when a promoter fragment is cloned upstream of the chloramphenicol resistance gene ribosome-binding site is this plasmid capable of conferring chloramphenicol resistance at levels greater than 5 μg/ml (21). Plasmid pMM6180 is a single-copy mini-pMccB17 composed of the pMccB17 replicon, the entire microcin region, and the Tc^r determinant from Tn*10* (O. Mayo and F. Moreno, unpublished construct). Plasmid pMM206-d8 is a Km^r pMM102 derivative with *mcbA* and the promoter located upstream of this gene deleted (29). All genetic manipulations were performed as described (22, 26). Liquid and solid LB and M63 media were prepared as described by Miller (26). Antibiotics and X-Gal were used at the same concentrations as we have described (12). Microcin activity was assayed as described (15), and β-galactosidase assays were performed as described by Miller (26). All values presented represent the mean of four determinations. All DNA manipulations, including restriction enzyme digests, ligations, and transformations, were performed as described (22). Maxicells of strain RYC1000 were prepared as described (30). Labeling with 50 μCi of [^{35}S]methionine per ml was performed for 10 min at 37°C. Labeled proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described (20).

DNA sequence analysis. The entire sequence of both strands was obtained by the chain termination method (31). Initially, after sonication of the MccB17 DNA, random fragments were cloned into the *Hinc*II site of M13mp8 (24). With the initial results from these clones, we constructed a

TABLE 1. Bacterial strains and plasmids

Strain	Genotype or characteristic	Reference or source
MC4100	F⁻ *araD139* Δ*lacU169 rpsL relA thiA*	(5)
RYC1000	MC4100; Δ*rib-7 recA56 gyrA*	(14)
RYC514	MC4100; *ompR101*	(16)
pop3001.6	MC4100; *malT*::Mu *c*ts	(16)
POI1681	*araD* Δ*(ara-leu)7697* Δ*(pro-lac) XIII rpsL* Mu *c*ts Mu d1(Km *lac*)	(6)
POII1681	*araD* Δ*(ara-leu)7697* Δ*(pro-lac) XIII rpsL* Mu *c*ts Mu d2(Km *lac*)	(6)
MH3497	Δ*lac gal rpsL* Mu *c*ts	(11)
Plasmid		
pMM102	pBR322 with *mcbABCDEF* genes	(28)
pMM206-d8	pMM102 with *mcbBCDEF* genes	(29)
pSS81	pMM102 *mcbA*::IS*1*	(29)
pSS11	pMM102 *mcbB*::IS*10*	(29)
pSS15	pMM102 *mcbC*::IS*10*	(29)
pSS33	pMM102 *mcbD*::IS*10*	(29)
pUC13	Ap^r Lacα$^+$	(25)
pACYC184	Cm^r Tc^r	(7)
pCID909	Cm^r MccB17$^+$ ImmB17$^+$	(12)
pSL100	Ap^r Cm^s promoter probe	(21)
pMM6180	Tc^r MccB17$^+$ ImmB17$^+$	This study

detailed restriction map. From this, we generated a number of specific clones into M13mp18 and M13mp19 to fill the gaps where confirming strand sequences had not been obtained. Both strands of the entire region presented were sequenced. The DNA sequences were arranged by using the GEL program (Intelligenetics).

Isolation of mini-Mu d fusions and determination of site of insertion. Strains POII1681(pMM102), POI1681(pCID909), and POII1681(pCID909) were used to produce transducing particles. These were used to infect MH3497 or pop3001.6 as described (6). The transduction mixture was incubated at 30°C for 1 h to allow expression of antibiotic resistance and then was plated on LB medium with kanamycin, X-Gal, and the appropriate antibiotic: chloramphenicol for pCID909 and ampicillin for pMM102 derivatives. Blue, drug-resistant colonies were tested for MccB17 production and immunity as described (12). Plasmid DNA was prepared by the SDS-alkali lysis. This DNA was directly annealed with a synthetic oligonucleotide primer complementary to the N-terminal segment of the *lacZ* gene present in mini-Mu d2. Sequencing was performed directly from the double-stranded template to determine the exact site of mini Mu d insertion.

S1 nuclease protection assays. RNA samples were prepared by the Triton X-100 method (9). Single 5′-end labeled probes were prepared as described (23). Total RNA (5 μg) and 0.1 μg of labeled probe in 30 μl of hydridization buffer (4) containing 80% formamide were boiled and then hybridized at 37°C for 3 h. A 270-μl sample of S1 buffer (4) containing 300 U of S1 (Boehringer Mannheim Biochemicals, catalog no. 818-348) was added, and the reaction was incubated for 1 h at 37°C. After ethanol precipitation, samples were suspended in 20 μl of formamide dyes, boiled, and electrophoresed in 6% polyacrylamide-6 M urea gels (23). G+A and C+T sequencing reactions were performed as described (23).

Primer extension assay. A 25-base synthetic oligonucleotide complementary to the mRNA from within the start of *mcbD* (Fig. 1, positions 2336 to 2360) was 5′ end labeled as described (23). Labeled primer (50,000 cpm) was hybridized to 50 μg of RNA in 30 μl of hybridization buffer (4) by denaturing at 95°C for 5 min and incubating at 43°C for 3 h. After ethanol precipitation, the hybridized RNA and primer were suspended in 30 μl of reverse transcriptase mix (1 mM each deoxynucleoside triphosphate, 1.25 μl of RNase inhibitor [Boehringer], 50 mM Tris [pH 8.3], 75 mM KCl, 10 mM dithiothreitol, and 3 mM $MgCl_2$). A total of 40 U of avian myeloblastosis virus reverse transcriptase (IBI) was added, and the mix was incubated at 43°C for 90 min. After treatment with DNase-free RNase A (100 μg/ml) for 30 min at 37°C, the cDNA was precipitated with ethanol, dissolved in 20 μl of formamide dye loading buffer, and electrophoresed.

RESULTS

Sequence of the MccB17 production region. The complete nucleotide sequence from the *Bam*HI site to the *Sal*I site was determined as described in Materials and Methods and is shown in Fig. 1. The locations of the previously determined restriction sites were confirmed by the sequence (28). However, there are two *Pst*I sites separated by 33 base pairs, where a single site had been mapped previously. The major open reading frames found in the sequence correlate well with the location of the complementation groups determined earlier, and their orientation is in agreement with transcription proceeding from left to right as suggested before (16).

To obtain direct evidence as to which of these reading frames were indeed translated into protein in vivo, we generated translational fusions using mini-Mu d2(Km *lac*). Mcc⁻Lac⁺ insertions into plasmids harboring the MccB17 production genes (pMM102 and pCID909 [see Table 1]) were isolated and mapped as described in Materials and Methods. The exact locations of insertion of 13 fusions are shown in Table 2 and schematically represented (see Fig. 4, line b). These fusions thus provide direct evidence that the open reading frames are translated regions corresponding to the four genes defined by complementation.

The AUG initiation codon of *mcbA* is preceded by the sequence AGGA, which is complementary to the 3′ end of the 16S rRNA and thus a good candidate for a ribosome-binding site (32). The end of *mcbA* and the first potential start site of *mcbB* are separated by 26 bases. The *mcbB* gene has two possible start sites at positions 643 and 676. Both of these are preceded by potential ribosome-binding sites. We have no direct evidence for determining which start codon is used. Nevertheless, the second start codon is more likely to be the one used primarily in vivo because the spacing between the ribosome-binding site and the AUG codon at position 676 is six bases while the spacing to the first start is only two bases.

The end of *mcbB* and the start of *mcbC* are separated by only one base, so that the Shine-Dalgarno sequence of *mcbC* is found within the coding region of *mcbB*. The open reading frames for *mcbC* and *mcbD* overlap by 20 bases. Within this area of overlap, there is an *Acc*I site (Fig. 1, position 2342). In our complementation analyses, we determined that a deletion up to that site resulted in an *mcbD* mutant genotype, while deletions up to the *Hin*dIII site located about 200 bases upstream (position 2122) were still *mcbD*⁺ (29). Since only the first of several in-frame AUG codons is removed in the *Acc*I deletion (29 [plasmid pMM138]), we conclude that this first initiation codon is used in vivo. Further evidence was obtained by cloning the *Acc*I fragment into pUC18 in the

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Bam HI
GGATCCACTTCAGCCGGAAACCGGATCTGCACGGCGGGAAACGGGCAAATGATATGATATGGAAGGCGGCACTCAGTTGTTGCATATGATACCGGCGTAA  100

GCCGGTACTGATTGACAGGTTTCACCTTACCCACCCCCGGCCCTGCCAGACCAGTGGGGGCTTAAAGGGGTAGTGTGACATCCTGACAGCAACTGAAAGC  200

AAAATGTAAACACAATATTAATTTTTGTAAAAAAACAATAACAGCATAAACATAACCAACTGATATTATTATTAAATATTTAACAGGCGTACAAATTTAG  300

TTCAAATTATCATTGCAAAATATAGTTAATTACGGCAAGTAACTAGTGTTGGCCAACATACTATTCAGATGTCATAAGCATTAATTTCCCTTAAAAAAGG  400

AGTCCTTATGGAATTAAAAGCGAGTGAATTTGGTGTAGTTTTGTCCGTTGATGCTCTTAAATTATCACGCCAGTCTCCATTAGGTGTTGGCATTGGTGGT  500
         METGluLeuLysAlaSerGluPheGlyValValLeuSerValAspAlaLeuLysLeuSerArgGlnSerProLeuGlyValGlyIleGlyGly

GGTGGCGGCGGCGGCGGCGGCGGTAGCTGCGGTGGTCAAGGTGGCGGTTGTGGTGGTTGCAGCAACGGTTGTAGTGGTGGAAACGGTGGCAGCGGCGGAA  600
GlyGlyGlyGlyGlyGlyGlyGlySerCysGlyGlyGlnGlyGlyGlyCysGlyGlyCysSerAsnGlyCysSerGlyGlyAsnGlyGlySerGlyGlyS

GTGGTTCACATATCTGATACGTTGAATTAACCGTTCAGGAGCATGGTGCTCCCTGATATTAAAAAAGGAAAAGATATGATCAATATTCTGCCGTTTGAGA  700
erGlySerHisIleTER                           METValLeuProAspIleLysLysGlyLysAspMETIleAsnIleLeuProPheGluI

                                                Acc I
TAATTTCCAGAAATACAAAGACCCTGCTTATTACCTACATTTCTTCAGTAGACATTACACATGAGGGAATGAAAAAAGTACTCGAAAGCCTACGATCCAA  800
leIleSerArgAsnThrLysThrLeuLeuIleThrTyrIleSerSerValAspIleThrHisGluGlyMetLysLysValLeuGluSerLeuArgSerLy

ACAGGGTATTATTTCTGAATACCTCCTTGATAAACTACTGGACGAATCGCTTATTGATAAAGACAAGGGCAAAGAGTTTCTTATTACCACAGGCGTGATA  900
sGlnGlyIleIleSerGluTyrLeuLeuAspLysLeuLeuAspGluSerLeuIleAspLysAspLysGlyLysGluPheLeuIleThrThrGlyValIle

                                                                              Sma I
AATAAAACGAAAACATCACCTCTCTGGGTAAACTCAGTCATAATAAGCGATGTTCCACATCTTTTCAGTAATGCCCGGGAACAATGGAAATGTGATGGTG  1000
AsnLysThrLysThrSerProLeuTrpValAsnSerValIleIleSerAspValProHisLeuPheSerAsnAlaArgGluGlnTrpLysCysAspGlyV

TTTTTGTTTCTCATATCATTGATATAAAAGATAATAATATTAATGTGAGCGATTCAACACTAATCTGGTTACATCTCGAAAACTATCATTCAGACATTGT  1100
alPheValSerHisIleIleAspIleLysAspAsnAsnIleAsnValSerAspSerThrLeuIleTrpLeuHisLeuGluAsnTyrHisSerAspIleVa

AAAAAGAATCTATTCAAAATTTGAAAGTAACCCTGGTGTTGCCTTCATCCAAAGCTACTACCTAAAGGAGTCATTCAGGATAGATGGAGTATATTCACCT  1200
lLysArgIleTyrSerLysPheGluSerAsnProGlyValAlaPheIleGlnSerTyrTyrLeuLysGluSerPheArgIleAspGlyValTyrSerPro

                                                               Hind III
GATCTTGGTACTCCCTGCCATTTTTGTCATATAGAGAGATGGCTGAGCAGGGAAGAAAAAAGCTTCAGACGTAACGAAATGTCCTGGGCAAACTTACTTC  1300
AspLeuGlyThrProCysHisPheCysHisIleGluArgTrpLeuSerArgGluGluLysSerPheArgArgAsnGluMetSerTrpAlaAsnLeuLeuG

AGTTACTGAAAAAATACCAAATGACACTACCAGCATTGGCTTTAGGCGAATCAGAAAGAGGATTCAGCTATCATTTAATAAAACGGCGACTTCAGGAGTT  1400
lnLeuLeuLysLysTyrGlnMetThrLeuProAlaLeuAlaLeuGlyGluSerGluArgGlyPheSerTyrHisLeuIleLysArgArgLeuGlnGluLe

GACAGGTACTTCACTGGTTAAAAGTCATGTCGATAACTTTATGTCATCTGTTAGCGCTGATTTAATCACCTGTATTTTATGTAAAGAGCCGGTAATTCAC  1500
uThrGlyThrSerLeuValLysSerHisValAspAsnPheMetSerSerValSerAlaAspLeuIleThrCysIleLeuCysLysGluProValIleHis

        Pvu II
TGGCAGGCTTGCAGCTGTCTGGAGAGATAACATGTCAAAACACGAACTCTCTTTAGTGGAAGTAACGCATTACACAGATCCTGAAGTTCTGGCCATTGTT  1600
TrpGlnAlaCysSerCysLeuGluArgTER METSerLysHisGluLeuSerLeuValGluValThrHisTyrThrAspProGluValLeuAlaIleVal

                                                                         Kpn I
AAAGATTTTCATGTCAGAGGTAACTTTGCTTCCCTCCCCGAATTTGCTGAACGAACTTTCGTGTCCGCGGTACCTCTTGCCCATCTGGAGAAATTTGAAA  1700
LysAspPheHisValArgGlyAsnPheAlaSerLeuProGluPheAlaGluArgThrPheValSerAlaValProLeuAlaHisLeuGluLysPheGluA

ATAAAGAAGTTCTCTTCAGGCCAGGTTTCAGCTCCGTAATAAACATATCCTCATCACATAATTTTAGTCGTGAAAGGCTCCCATCAGGAATAAACTTTTG  1800
snLysGluValLeuPheArgProGlyPheSerSerValIleAsnIleSerSerSerHisAsnPheSerArgGluArgLeuProSerGlyIleAsnPheCy

CGACAAAAATAAACTTTCCATTCGTACTATTGAAAAGTTATTAGTCAATGCATTCAGCTCACCTGATCCTGGCTCTGTAAGAAGACCTTATCCTTCTGGG  1900
sAspLysAsnLysLeuSerIleArgThrIleGluLysLeuLeuValAsnAlaPheSerSerProAspProGlySerValArgArgProTyrProSerGly

GGGGCATTGTACCCGATTGAAGTTTTTTTATGCAGATTATCTGAAAATACAGAAAACTGGCAGGCAGGAACTAATGTTTATCACTACCTGCCGCTAAGTC  2000
GlyAlaLeuTyrProIleGluValPheLeuCysArgLeuSerGluAsnThrGluAsnTrpGlnAlaGlyThrAsnValTyrHisTyrLeuProLeuSerG
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correct orientation and showing that it was unable to complement *mcbD* insertion mutations in *trans*. It should be noted that a deletion removing the last two codons of *mcbC* is phenotypically McbC$^+$ (29 [plasmid pMM137]). Indeed, pMM137 encodes a McbC-McbF protein fusion of 422 amino acids in which only the last McbC residue (Gln) is missing and replaced with a Pro residue from within McbF (12).

The predicted sizes for the gene products are as follows: McbA (69 amino acids), 6,013 daltons; McbB (284 or 295 amino acids), 32,764 or 33,989 daltons; McbC (272 amino

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AGGCACTGGAACCTGTTGCTACATGTAATACTCAGTCACTCTACCGAAGCCTGTCCGGTGGGGATTCGGAACGTCTTGGTAAACCCCATTTTGCTCTCGT  2100
lnAlaLeuGluProValAlaThrCysAsnThrGlnSerLeuTyrArgSerLeuSerGlyGlyAspSerGluArgLeuGlyLysProHisPheAlaLeuVa

                   Hind III
CTATTGCATTATTTTTGAAAAAGCTTTGTTCAAATATCGCTACAGAGGATATCGGATGGCCTTAATGGAAACAGGTTCGATGTATCAGAACGCAGTATTG  2200
lTyrCysIleIlePheGluLysAlaLeuPheLysTyrArgTyrArgGlyTyrArgMetAlaLeuMetGluThrGlySerMetTyrGlnAsnAlaValLeu

GTTGCAGATCAAATAGGACTGAAAAACCGGGTATGGGCGGGATATACCGATTCATACGTAGCAAAAACAATGAATCTGGATCAGAGGACTGTAGCGCCAC  2300
ValAlaAspGlnIleGlyLeuLysAsnArgValTrpAlaGlyTyrThrAspSerTyrValAlaLysThrMetAsnLeuAspGlnArgThrValAlaProL

                                          Acc I
TGATCGTTCAGTTTTTTGGAGATGTAAACGATGATAAATGTCTACAGTAACCTTATGTCCGCATGGCCGGCCACAATGGCCATGAGTCCAAAACTGAACA  2400
euIleValGlnPhePheGlyAspValAsnAspAspLysCysLeuGlnTER
                                    METIleAsnValTyrSerAsnLeuMetSerAlaTrpProAlaThrMetAlaMetSerProLysLeuAsnA

GAAATATGCCAACGTTTTCTCAGATATGGGACTATGAGCGTATTACACCAGCCAGCGCGGCCGGTGAAACTCTGAAGTCAATTCAGGGGGCAATAGGTGA  2500
rgAsnMetProThrPheSerGlnIleTrpAspTyrGluArgIleThrProAlaSerAlaAlaGlyGluThrLeuLysSerIleGlnGlyAlaIleGlyGl

ATATTTTGAACGCCGTCATTTTTTTAATGAGATAGTCACCGGTGGTCAGAAAACATTATATGAGATGATGCCTCCATCTGCTGCAAAGGCTTTTACCGAA  2600
uTyrPheGluArgArgHisPhePheAsnGluIleValThrGlyGlyGlnLysThrLeuTyrGluMetMetProProSerAlaAlaLysAlaPheThrGlu

          Bgl II
GCATTTTTTCAGATCTCATCACTGACCCGCGATGAAATCATAACCCATAAATTTAAAACGGTCAGAGCCTTTAATCTGTTTAGCCTTGAACAACAAGAAA  2700
AlaPhePheGlnIleSerSerLeuThrArgAspGluIleIleThrHisLysPheLysThrValArgAlaPheAsnLeuPheSerLeuGluGlnGlnGluI

   Pst I                                  Pst I
TACCTGCAGTCATAATTGCACTCGACAATATAACCGCTGCAGATGATCTGAAATTTTATCCTGACAGAGATACATGCGGATGTAGCTTTCATGGTAGTTT  2800
leProAlaValIleIleAlaLeuAspAsnIleThrAlaAlaAspAspLeuLysPheTyrProAspArgAspThrCysGlyCysSerPheHisGlySerLe

GAACGATGCCATAGAAGGTTCCTTGTGTGAATTTATGGAGACACAGTCCCTCCTTCTTTACTGGTTACAGGGAAAAGCCAATACTGAAATATCCAGTGAA  2900
uAsnAspAlaIleGluGlySerLeuCysGluPheMetGluThrGlnSerLeuLeuLeuTyrTrpLeuGlnGlyLysAlaAsnThrGluIleSerSerGlu

ATAGTAACAGGCATAAATCATATAGATGAGATTTTACTGGCTCTCAGGTCAGAAGGAGATATCAGGATTTTCGATATCACCCTGCCCGGAGCTCCTGGAC  3000
IleValThrGlyIleAsnHisIleAspGluIleLeuLeuAlaLeuArgSerGluGlyAspIleArgIlePheAspIleThrLeuProGlyAlaProGlyH

ACGCAGTACTAACCCTGTATGGCACAAAAAACAAAATCAGTCGAATAAAATACAGTACCGGATTATCCTATGCTAATAGTCTGAAAAAAGCACTTTGTAA  3100
isAlaValLeuThrLeuTyrGlyThrLysAsnLysIleSerArgIleLysTyrSerThrGlyLeuSerTyrAlaAsnSerLeuLysLysAlaLeuCysLy

ATCCGTAGTGGAATTGTGGCAATCGTATATATGCCTGCACAACTTTCTTATTGGCGGTTATACTGATGATGACATTATTGATAGTTACCAGCGTCACTTT  3200
sSerValValGluLeuTrpGlnSerTyrIleCysLeuHisAsnPheLeuIleGlyGlyTyrThrAspAspAspIleIleAspSerTyrGlnArgHisPhe

ATGTCATGCAACAAGTACGAGTCGTTTACGGATTTGTGTGAAAATACGGTACTACTGTCTGATGATGTCAAGTTAACGCTTGAGGAAAATATTACGTCAG  3300
MetSerCysAsnLysTyrGluSerPheThrAspLeuCysGluAsnThrValLeuLeuSerAspAspValLysLeuThrLeuGluGluAsnIleThrSerA

ACACAAATTTATTAAACTATCTTCAACAAATTTCTGATAATATTTTTGTTTACTATGCCAGGGAAAGAGTAAGTAACAGCCTTGTCTGGTACACAAAAAT  3400
spThrAsnLeuLeuAsnTyrLeuGlnGlnIleSerAspAsnIlePheValTyrTyrAlaArgGluArgValSerAsnSerLeuValTrpTyrThrLysIl

AGTAAGCCCTGATTTTTTCCTTCATATGAATAACTCAGGTGCAATAAACATTAATAATAAAATTTACCATACCGGGGACGGTATTAAAGTCAGAGAATCA  3500
eValSerProAspPhePheLeuHisMetAsnAsnSerGlyAlaIleAsnIleAsnAsnLysIleTyrHisThrGlyAspGlyIleLysValArgGluSer

    Kpn I
AAGATGGTACCATTCCCATAAGGATAACAAATGGTTACATTAAAAATGGCGATAATTTTTCTGATGGAACAAATCAGAACCCCTTTCAGCTTAATATGGA  3600
LysMetValProPheProTER            METValThrLeuLysMetAlaIleIlePheLeuMetGluGlnIleArgThrProPheSerLeuIleTrpT

CAATTATGTCACCTACAGTGTTGTTCTTTTTCCTTCATTTCAATGAAATTGAACTTCATTATGGTGATACAGCATGGCTCGGAAAACAAATATCATGGTT  3700
hrIleMetSerProThrValLeuPhePhePheLeuHisPheAsnGluIleGluLeuHisTyrGlyAspThrAlaTrpLeuGlyLysGlnIleSerTrpPh

TGTAGGCTACATTTCTTTTTCTGTTGTTCTGTTTAATTACTGTCTGTATCTGGTCGGAAGAAGAGAAAGTGGTTTTATTGCTACCTTCGTGCATAATATG  3800
eValGlyTyrIleSerPheSerValValLeuPheAsnTyrCysLeuTyrLeuValGlyArgArgGluSerGlyPheIleAlaThrPheValHisAsnMet

   Sal I
GATGGTCGAC  3810
AspGlyArg
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FIG. 1. Nucleotide sequence of the MccB17 production genes. The nucleotide sequence of the sense strand for the four genes is presented along with the predicted amino acid sequence of the four products. Some of the key sites used for constructions and reference are indicated.

acids), 30,757 daltons; and McbD (396 amino acids), 44,870 daltons.

Identification of the *mcb* gene products. The product of *mcbA* has been identified previously by using minicells (10). To determine the sizes of the gene products synthesized from the other *mcb* genes, we utilized maxicells labeled with [^{35}S]methionine. Maxicells with the MccB17-producing plasmid pMM102 are not presented because *recA* (pMM102)

TABLE 2. Translational fusions in the MccB17 production genes

Plasmid and fusion no.	Nucleotide of insertion	Gene and insertion[a]	Activity[b]
pMM102			
498	423	*mcbA* Ser-6	
10	676	*mcbB* Met-1	
224	792	*mcbB* Arg-40	
244	1068	*mcbB* Leu-132	
470	1504	*mcbB* Glu-277	
228	1862	*mcbC* Pro-111	880
548	1877	*mcbC* Val-116	910
6	2180	*mcbC* Met-217	3,340
pCID909			
11.25	2180	*mcbC* Met-217	750
18.2	2361	*mcbD* Ala-11	1,140
18.5	2622	*mcbD* Leu-98	150
33.21	2949	*mcbD* Ser-207	100
pMM102			
483	2370	*mcbD* Ala-14	6,560

[a] The codon position is indicated. For *mcbB*, the second potential start site is used as Met-1.

[b] β-Galactosidase activity was determined from exponential (optical density at 600 nm equals 0.5) cultures of pop3001.6 harboring the different plasmids grown at 30° in minimal medium. Activity is expressed in Miller units (26).

strains do not grow in minimal medium due to their defective immunity (12, 13). The identification of products from *mcbB* and *mcbD* in maxicells was possible from pMM102 derivatives containing insertions in these genes. These previously described plasmids (29) are listed in Table 1 and shown in Fig. 2A. $mcbB^+$ plasmids give a product migrating at 29,500 daltons which disappears when *mcbB* is mutated (Fig. 3A, lane 4 [pSS11]). Similarly, the *mcbD* product was identified as a 45,500-dalton protein which disappears in *mcbD* mutants (Fig. 3A, lane 6 [pSS33]). It should be noted that McbD is unstable. Following a 30-min chase, no McbD product could be identified in maxicells (results not shown).

Because of its instability, McbD could not always be identified in the plasmids described above. To identify this product unambiguously, we cloned *mcbD* and the *mcbD* mutant insertion present in pSS33 into pUC13 so that they would be expressed from the *lac* promoter (Fig. 2B). This generated plasmids pMM5066 and pMM5332, respectively. Lanes 8 and 9 of Fig. 3A confirm that the 45,500-dalton protein is indeed the product of *mcbD*. Results identical to those described above were obtained with several other mutations in *mcbB* and *mcbD* (data not shown). The sizes of the products are in agreement with those predicted from the nucleotide sequence.

Maxicells carrying pMM102 derivatives with *mcbC* mutations did not seem to lack any band which could be identified as McbC. The predicted size of McbC is 30,757 daltons, a size very close to that of the *bla* gene product. Thus, McbC could be masked by β-lactamase. To clone the *mcbC* gene in a plasmid lacking the *bla* gene, we first subcloned the fragment spanning from *Pvu*II (position 1515) to *Bgl*II (position 2612) (Fig. 1) into the *Hinc*II and *Bam*HI sites of pUC13 to generate pMM5060 (Fig. 2C). From this construct, we further subcloned the *Pvu*II-*Eco*RI fragment containing the *mcbC* gene under the control of the *lac* promoter into the *Pvu*II and *Eco*RI sites of pACYC184. This plasmid was designated pMM5067 (Fig. 2C). Four different IS*10* insertions into *mcbC* were similarly subcloned into pACYC184 to generate pMM5015, pMM5022, pMM5035, and pMM5038 (Fig. 2C). Figure 3B shows the products synthesized in maxicells harboring the *mcbC* subclone pMM5067 in lane 2 and one *mcbC* mutation, pMM5015, in lane 3. The three other insertions gave identical results (data not shown). The difference observed is the disappearance of a band of about 31,000 daltons in the mutants. This fits well with the predicted size for McbC and confirms the hypothesis that the product can be masked by β-lactamase.

Transcription of the MccB17 production genes. The arrangement of the four genes involved in MccB17 production suggests that these genes are transcribed as an operon. The largest intergenic space is found between *mcbA* and *mcbB*, and it is either 25 or 58 base pairs, depending on the exact start site of *mcbB*. In this region, there is a terminator of transcription, but readthrough transcripts can proceed into *mcbB* (Z. Han and R. Kolter, manuscript in preparation). The intergenic space between *mcbB* and *mcbC* is only 1 base pair, and *mcbC* and *mcbD* actually overlap by 20 base pairs. This is shown schematically in Fig. 4, line a. However, previous results from complementation studies and transcriptional fusions suggested the possibility that multiple promoters are involved in the transcription of these genes (16, 29). In particular, three observations were consistent with our interpretation of multiple promoters. First, we observed no clear polarity effects from a large number of insertion mutations within these four genes. Second, expression of transcriptional fusions of *lacZ* inserted in *mcbA*, *mcbB*, and *mcbC* increased during stationary phase while expression was growth phase independent for a *lacZ* fusion in *mcbD*. We have identified a promoter upstream of *mcbA* (P_{mcb}) whose expression is induced after the cessation of growth (8). This promoter could be responsible for the transcription of *mcbA*, *mcbB*, and *mcbC*. However, it is not clear how it could transcribe *mcbD* given the growth phase-independent expression of the fusion in *mcbD*.

To investigate the contribution of P_{mcb} to the expression of *mcbC* and *mcbD*, we placed the terminator cassette omega (27) in the *Sma*I site within the *mcbB* gene of four plasmids containing Mu d2 fusions. Fusions 228, 548, and 6 are in *mcbC*, while 483 is in *mcbD*. The exact locations of these fusions are given in Table 2, and the omega cloning is diagrammed in Fig. 4, lines b and c. The levels of β-galactosidase in these plasmids, with and without the omega terminator, were determined and are shown in Table 3. The presence of omega almost completely abolished *lacZ* expression in fusions in *mcbC*, suggesting that P_{mcb} is the major promoter for *mcbC*. Surprisingly, P_{mcb} also influences greatly the expression of *mcbD*. There is close to a 10-fold reduction in the levels of *lacZ* expression from fusion 483 when omega is present. The residual activity does not show any dependence on the OmpR product. These results suggested the presence of a weaker secondary promoter located within *mcbC* but downstream of the site of insertion of mini-Mu d2 in fusion 548. To identify the location of the promoter responsible for this residual transcription, we mapped the 5′ end of the mRNA as described in Materials and Methods. RNA was prepared from a strain harboring pMM102 with the *mcbD*-483 fusion and the omega terminator in the *Sma*I site. Figure 5 shows a cDNA of 113 bases, corresponding to a start site located in position 2248, 83 base pairs upstream of the *mcbD* start. An unusual −10 sequence (CGGGAT) is found 4 base pairs upstream and, spaced by 17 base pairs, is a relatively close-to-consensus −35 sequence (AGGACT) (see Discussion).

Leftward transcription of the *mcb* genes. All the mini-Mu d2 (Km *lac*) translational fusions obtained in the four MccB17 production genes are transcribed left to right as shown (Fig. 4, line b). This is consistent with our findings

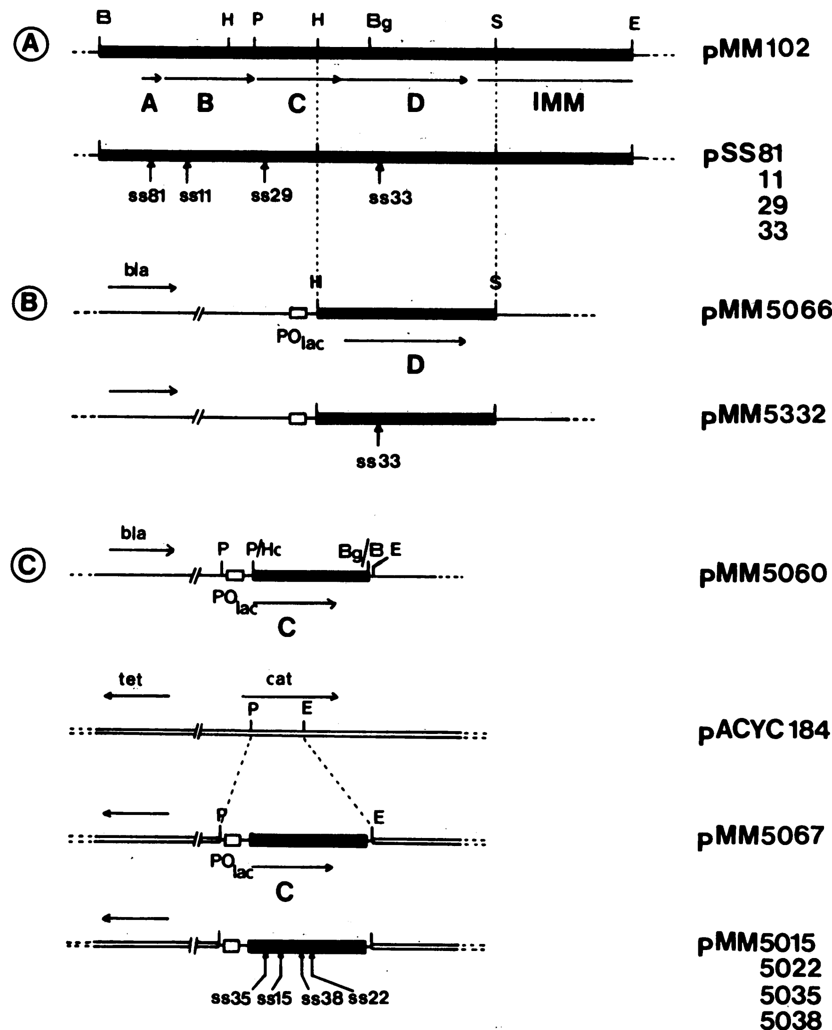

FIG. 2. Structures of plasmids used in the identification of *mcb* gene products. (A) Restriction map of the MccB17 DNA inserted into pBR322 in pMM102 and locations of the insertions in the derivatives pSS81, pSS11, pSS29, and pSS33. (B) Cloning of *mcbD* and an insertion derivative into pUC13 to generate pMM5066 and pMM5332. (C) The two-step cloning of *mcbC* and insertion derivatives in a β-lactamase-free vector, pACYC184, to generate pMM5067, pMM5015, pMM5022, pMM5035, and pMM5038. Abbreviations: B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; S, *Sal*I; E, *Eco*RI; Hc, *Hin*cII; P, *Pvu*II.

from the sequence that all major open reading frames are transcribed and translated in the same orientation. However, leftward transcription in the central region of the *mcb* genes was suggested by several results. First, the Km^r and Ble^r determinants from Tn*5* lacking a promoter were expressed when cloned in the *Bgl*II site within *mcbD*, independent of orientation (14). Second, in the wild-type plasmid we obtained an active Mu d1(Ap *lac*) insertion within *mcbC* oriented to transcribe from right to left (16). This fusion expressed β-galactosidase at low levels and did not show growth phase regulation. Even though we analyzed dozens of mini-Mu d2(Km *lac*) translational fusions, none was transcribed from right to left. Therefore, we isolated mini-Mu d1(Km *lac*) transcriptional fusions oriented such that transcription was from right to left. We used pCID909 (12), a pACYC184 derivative harboring all the MccB17 production and immunity genes, as the target plasmid. Indeed we found several such fusions. We mapped the site of insertion of four of these using restriction endonucleases. Three of them were found within *mcbC* and one just to the right of the *Bgl*II site within *mcbD* (Fig. 4, line d). We then measured the levels of β-galactosidase from these fusions in the multicopy plasmid in which they were isolated. The levels in MC4100 grown in minimal medium were 352, 341, 362, and 435 Miller units for fusions 2.11, 2.12, 3.7, and 3.8, respectively.

We then wanted to determine the exact position of the leftward promoter responsible for this transcription. To this end, we focused our efforts on the *Sau*3AI fragment present within *mcbD* and spanning from the *Bgl*II site (position 2612) to position 2745. This fragment was chosen because it is located just to the right of the rightmost insertion, 2.11. This fragment was cloned into the *Bam*HI site of the promoter probe plasmid pSL100 (21) in the appropriate orientation for the chloramphenicol resistance gene to be expressed, i.e., such that the half-*Bgl*II site is proximal to the start of chloramphenicol resistance gene translation. Cells harboring this plasmid, designated pOG5502, are resistant to 350 μg of chloramphenicol per ml.

We then attempted to identify the 5′ end of transcripts initiating from this region in the wild-type plasmid pMccB17 by S1 nuclease protection analysis. RNA was prepared from exponential and stationary-phase cells harboring pMccB17

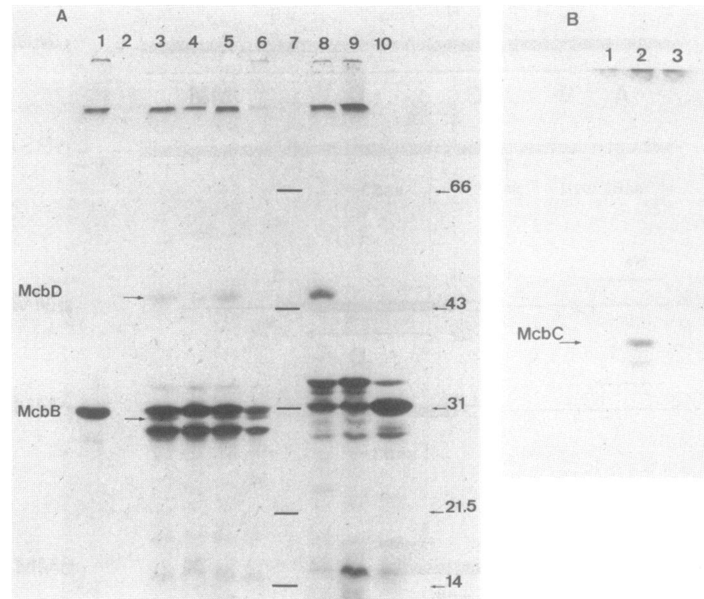

FIG. 3. (A) Identification of McbB and McbD. Maxicells were prepared, labeled, and run in SDS-polyacrylamide gel electrophoresis as described in Materials and Methods (11.5% gel). All cells are RYC1000 harboring. Lanes: 1, pBR322; 2, no plasmid; 3, pSS81; 4, pSS11; 5, pSS29; 6, pSS33; 8, pMM5066; 9, pMM5332; 10, pUC13. Lane 7 contained molecular weight standards which are denoted with the horizontal bars. The sizes in kilodaltons are shown on the right side. The McbB and McbD products are indicated with arrows from the left side. (B) Identification of McbC by 13% SDS-polyacrylamide gel electrophoresis. Maxicells of RYC1000 harbored in lanes as follows: 1, no plasmid; 2, pMM5067; 3, pMM5015.

and was probed with two different end-labeled fragments spanning the *Bgl*II site. In both cases, we were unable to detect a signal. We then prepared RNA from cells harboring the promoter probe construction pOG5502. The probe in this case was a fragment from pOG5502 labeled at a *Dde*I site present in the vector pSL100. The probe spanned the entire cloned *Sau*3AI fragment and ended in a *Bgl*II site also present in the vector pSL100. The results of this S1 mapping are shown in Fig. 6. Leftward transcription from this third promoter, designated P_3, starts at position 2630. The sequence immediately upstream of the start site shows a good fit with the consensus sequence for sigma-70 promoters in *E. coli* (Fig. 7).

DISCUSSION

In this paper, we present the nucleotide sequence of the genes involved in the production of the polypeptide antibiotic MccB17. We also present the exact sites of fusion of 13 active translational fusions as direct evidence that the four major open reading frames in the sequence are translated into proteins. The product of the first gene, *mcbA*, was previously identified by using minicells (10). This product was also shown to be processed to yield the active molecule. The three other gene products, McbB, McbC, and McbD, were identified from results presented here. The molecular weights predicted from the nucleotide sequence correlated well with those observed for the gene products produced in maxicells as determined in SDS-polyacrylamide gels. The location of the four open reading frames in the sequence also correlates well with results obtained previously from genetic complementation (29).

The predicted amino acid sequences of the *mcbABCD* products were analyzed for their content of potential transmembrane domains by using the hydrophobicity values of Kyte and Doolittle (19). The results (data not shown) suggest that none of these proteins have membrane-spanning domains, but these results must be interpreted cautiously since some membrane-spanning domains are not predicted by these criteria (33). None of the four proteins has a characteristic signal peptide. Nonetheless, the *mcbA* product is exported as the active MccB17 molecule. We have shown that 26 amino acids are removed from the N terminus in the processing of the primary product (10). The lack of signal sequences and potential transmembrane domains in McbB, McbC, and McbD suggests that they are all localized in the cytoplasm. Elsewhere we have presented results indicating that the products of the two genes immediately downstream of *mcbD*, designated *mcbE* and *mcbF*, are in part responsible for the export of MccB17 (12). It is therefore likely that the roles of McbB, McbC, and McbD in the production of MccB17 are played in the cytoplasm of the producing cells. Exactly what those roles are remains to be determined, but we have argued that they are involved in steps prior to or during the removal of the 26 N-terminal amino acid residues of McbA (10). It should be noted that searches of the EMBL and GenBank data bases revealed no significant homologies between these products and published sequences.

Several observations suggest that the transcription of *mcbABCD* is regulated in a complex fashion which could involve several promoters in various regions of the cluster of genes. First, insertion mutations do not show polarity in complementation experiments (29). Second, while all four genes are regulated by the OmpR activator, only fusions to the first three genes increase in expression upon the onset of stationary phase (16). The simplest interpretation of these results is that there are two *ompR*-dependent promoters; one transcribes *mcbABC* and is subject to growth phase regulation, the other transcribes *mcbD* and is expressed at constant levels throughout growth. Third, active transcriptional fusions were isolated in both orientations within the *mcbC*

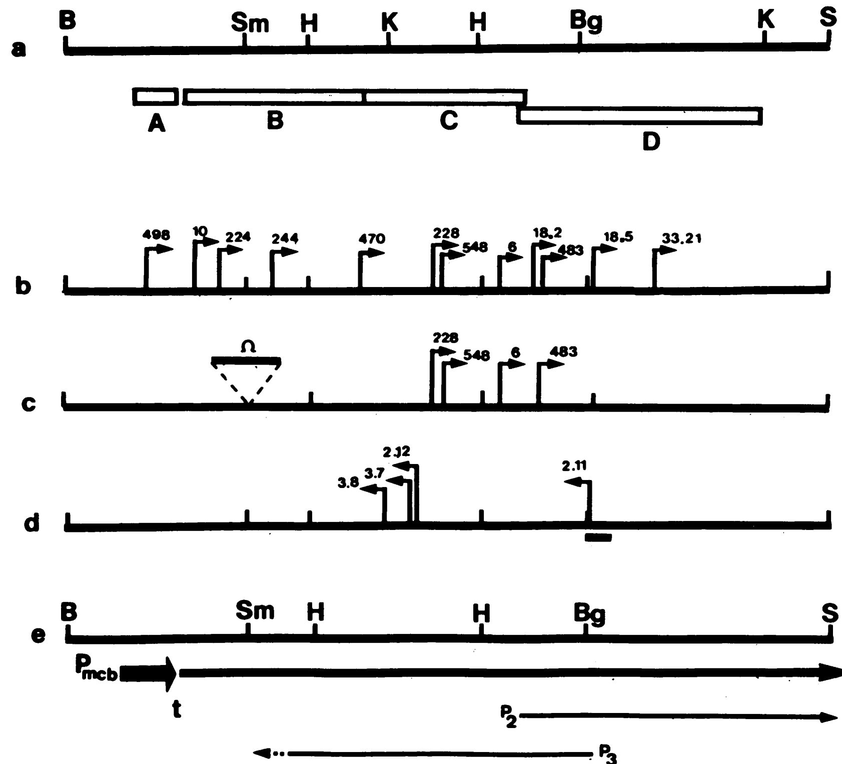

FIG. 4. Transcriptional pattern of the MccB17 production genes. (a) The alignment of the four production genes relative to key restriction sites. (b) The locations and orientations of mini-Mu d2 fusions within the production region. (c) The fusions that were tested for the effects of the omega terminator inserted at the *SmaI* site. (d) Locations of mini-Mu d1 fusions obtained which transcribe in the opposite direction from the mini-Mu d2 fusions. The *Sau*3AI fragment that contains a leftward promoter is shown by the bold line. (e) The overall transcriptional pattern of the *mcb* production genes. The major promoter, P_{mcb}, transcribes from upstream of *mcbA*. There is a terminator of transcription between *mcbA* and *mcbB* (Han and Kolter, in preparation). Readthrough transcription proceeds into *mcbB*, *mcbC*, and *mcbD*. Within *mcbC* there is a secondary promoter, P_2, transcribing *mcbD*. Within *mcbD* there is a promoter, P_3, transcribing in the opposite direction.

gene (16). These findings led us to identify and characterize the promoters responsible for the transcription of these genes.

Between the *Bam*HI site and the start of *mcbA* there is an *ompR*-dependent promoter, P_{mcb}, which transcribes rightward (16). We have identified the start site of P_{mcb} by using S1 nuclease protection: transcription starts at position 345 (8). By inserting a strong transcriptional terminator within the *mcbB* gene, we were able to determine the contribution of P_{mcb} to the transcription of *mcbC* and *mcbD*. Almost all of the transcription across *mcbC* (97 to 98%) was due to the P_{mcb} promoter. In the case of *mcbD*, it appears that about 90% of its transcription is also controlled by P_{mcb}. With a primer extension assay, we identified an internal promoter

TABLE 3. β-Galactosidase levels in fusions with and without omega in *ompR*$^+$ and *ompR* backgrounds[a]

Fusion	*ompR*$^+$		*ompR*
	Without omega	With omega	With omega
mcbC-228	980	20	10
mcbC-548	945	22	16
mcbC-6	2320	77	75
mcbD-483	5560	453	425

[a] Expressed in Miller units (26). Strains used were *ompR*$^+$ MC4100 and *ompR* RYC514. Growth was in minimal medium.

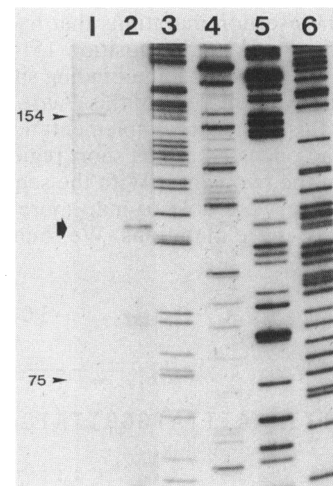

FIG. 5. Location of the start site for the *mcbD* promoter, P_2. Primer extension was done as described in Materials and Methods. Lanes: 1, pBR322 end-labeled *Hin*f fragments as size standards; 2, synthesized cDNA; 3 through 6, A, C, G, and T sequencing reactions from M13mp18 (24) using the 17-mer universal primer.

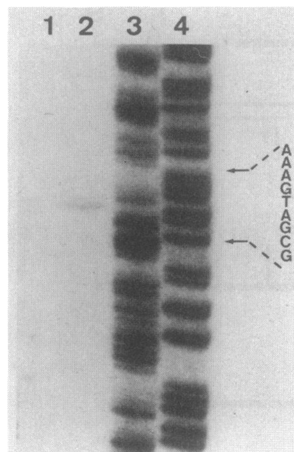

FIG. 6. Location of the RNA start site for the leftward promoter within *mcbD*. S1 mapping was done as described in Materials and Methods. Lanes: 1, tRNA control; 2, RNA isolated from cells harboring pOG5502; 3, C+T residues (23); 4, A+G residues (23).

which was responsible for the residual levels of *mcbD* transcription in the absence of P_{mcb} (P_2 in Fig. 4). The mRNA start site for P_2 is at nucleotide 2248. The -10 region of this promoter shows little similarity with the consensus -10 of sigma-70 promoters in *E. coli*. The -35 region, however, showed a relatively good match to consensus. In this respect, P_2 is similar to P_{mcb} (8).

Our results indicate that most of the expression of *mcbD* was due to P_{mcb}, a promoter that was induced during stationary phase. Thus, the lack of induction in stationary phase of a *lacZ* transcriptional fusion in *mcbD* remains a paradox (16). A possible explanation for this observation may be that there is differential stability among the various parts of the *mcb* mRNA. Experiments to determine the amount and stability of RNA from the various genes in cells harboring the wild-type plasmid are currently in progress.

A related issue when discussing the contribution of P_{mcb} to the transcription of downstream genes is the existence of a group of insertion mutations that fail to complement other mutations in both *mcbB* and *mcbC* (29). This aberrant group includes two IS*1* insertion mutations that had been mapped very close to the *Pvu*II site at position 1514. The last five codons of *mcbB* and the ribosome-binding site for *mcbC* are located immediately to the right of this *Pvu*II site. A possible explanation for this aberrant complementation group is that the insertions had occurred in this short region of functional overlap between the two genes. With the sequence information completed, we were able to map more accurately the site of insertion of these mutations. We found that they are located in the region of *mcbB* about 100 bases upstream of the *Pvu*II site. Thus, the more likely explanation for the behavior of these mutations is that these IS*1* insertions in *mcbB* show polarity on the expression of *mcbC*. Indeed, IS*1* insertions are usually polar (18). This polarity does not affect *mcbD* because of the existence of P_2. In contrast, the lack of polarity of the IS*10* insertions could be due to transcripts proceeding from the outward promoter of this insertion sequence.

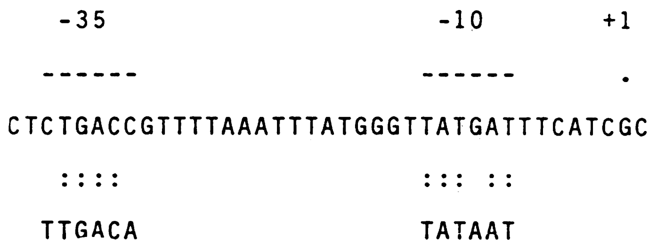

FIG. 7. Fit of sequence upstream of RNA start site for leftward promoter with consensus sequence for sigma-70 promoters in *E. coli*.

Finally, there appears to be an antisense RNA transcript from the MccB17 production genes. Supporting this is the finding that transcriptional but not translational fusions can be obtained in the opposite orientation to the translation of *mcbB*, *mcbC*, and *mcbD*. We mapped the start site for this transcript, thus defining a third promoter in this region. This promoter was designated P_3, but its transcript could only be detected in cells harboring this promoter (which is found within the coding region of *mcbD*) in a high-copy plasmid. However, Mu d1(Ap *lac*) fusions to this promoter expressed low but measurable levels of activity in the low-copy wild-type plasmid pMccB17. To determine if this counter transcript plays a regulatory role in the expression of the MccB17 production genes, the multicopy plasmid pMM206-d8 was introduced into cells carrying a copy of different *mcb-lacZ* fusions in the chromosome. In no case was the activity of the fusion altered by the presence of the plasmid. This suggests that the antisense RNA does not play a physiological role in the expression of the *mcb* production genes in *trans*. It remains to be determined if it has any effect in *cis*.

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LITERATURE CITED

1. **Asensio, C., J. C. Perez-Díaz, M. C. Martínez, and F. Baquero.** 1976. A new family of low molecular weight antibiotics from Enterobacteria. Biochem. Biophys. Res. Commun. **69:**7–14.
2. **Baquero, F., D. Bouanchaud, M. C. Martínez, and C. Fernández.** 1978. Microcin plasmids: a group of extrachromosomal elements coding for low-molecular-weight antibiotics in *Escherichia coli*. J. Bacteriol. **135:**342–347.
3. **Baquero, F., and F. Moreno.** 1984. The microcins. FEMS Microbiol. Lett. **23:**117–124.
4. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA **75:**1274–1278.
5. **Casadaban, M.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *E. coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:**541–555.
6. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158:**488–495.
7. **Chang, A. C. Y., and S. N. Cohen.** 1977. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15a cryptic miniplasmid. J. Bacteriol. **134:** 114–119.
8. **Connell, N., Z. Han, F. Moreno, and R. Kolter.** 1987. An *E. coli* promoter induced by the cessation of growth. Mol. Microbiol.

1:195–201.

9. **Cooper, T. G., P. Whitney, and B. Magasanik.** 1974. Reaction of *lac*-specific ribonucleic acid from *E. coli* with *lac* deoxyribonucleic acid. J. Biol. Chem. **249:**6548–6555.
10. **Davagnino, J., M. Herrero, D. Furlong, F. Moreno, and R. Kolter.** 1986. The DNA replication inhibitor microcin B17 is a forty-three-amino acid protein containing sixty percent glycine. Proteins **1:**230–238.
11. **Engebrecht, J., K. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773–781.
12. **Garrido, M. C., M. Herrero, R. Kolter, and F. Moreno.** 1988. The export of the DNA replication inhibitor microcin B17 provides immunity for the host cell. EMBO J. **7:**1853–1862.
13. **Garrido, M. C., J. L. San Millán, and F. Moreno.** 1986. A novel plasmid vector allowing positive selection for cloned fragments. FEMS Microbiol. Lett. **34:**261–264.
14. **Genilloud, O., M. C. Garrido, and F. Moreno.** 1984. The transposon Tn*5* carries a bleomycin resistance determinant. Gene **32:**225–232.
15. **Hernández-Chico, C., M. Herrero, M. Rejas, J. L. San Millán, and F. Moreno.** 1982. Gene *ompR* and regulation of microcin 17 and colicin E2 synthesis. J. Bacteriol. **152:**897–900.
16. **Hernández-Chico, C., J. L. San Millán, R. Kolter, and F. Moreno.** 1986. Growth phase and OmpR regulation of transcription of microcin B17 genes. J. Bacteriol. **167:**1058–1065.
17. **Herrero, M., and F. Moreno.** 1986. Microcin B17 blocks DNA replication and induces the SOS system in *Escherichia coli*. J. Gen. Microbiol. **132:**393–402.
18. **Iida, S., J. Meyer, and W. Arber.** 1983. Prokaryotic IS elements. *In* J. A. Shapiro (ed.), Mobile genetic elements. Academic Press, Inc., New York.
19. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227:**680–685.
21. **Li, S. C., C. L. Squires, and C. Squires.** 1984. Antitermination of *E. coli* rRNA transcription is caused by a control region segment containing lambda nut-like sequences. Cell **38:**851–860.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
23. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
24. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
25. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double digest restriction fragments. Gene **19:**269–276.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
28. **San Millán, J. L., C. Hernández-Chico, P. Pereda, and F. Moreno.** 1985. Cloning and mapping of the genetic determinants for microcin B17 production and immunity. J. Bacteriol. **163:**275–281.
29. **San Millán, J. L., R. Kolter, and F. Moreno.** 1985. Plasmid genes required for microcin B17 production. J. Bacteriol. **163:**1016–1020.
30. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137:**692–693.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
33. **Wallace, B. A., M. Cascio, and D. L. Mielke.** 1986. Evaluation of methods for the prediction of membrane protein secondary structure. Proc. Natl. Acad. Sci. USA **83:**9423–9427.