

## Growth-Phase-Dependent Expression of the Cyclopeptide Antibiotic Microcin J25

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**Microcin J25 is a 2,107-Da, plasmid-encoded, cyclopeptide antibiotic produced by *Escherichia coli*. We have isolated *lacZ* fusions to *mcjA* (encoding the 58-amino-acid microcin precursor) and *mcjB* and *mcjC* (which are required for microcin maturation), and the regulation of these fusions was used to identify factors that control the expression of these genes. The *mcjA* gene was found to be dramatically induced as cells entered the stationary phase. Expression of *mcjA* could be induced by resuspending uninduced exponential-phase cells in spent supernatant obtained from an early-stationary-phase culture. Induction of *mcjA* expression was not dependent on high cell density, pH changes, anaerobiosis, or the buildup of some inducer. A starvation for carbon and inorganic phosphate induced *mcjA* expression, while under nitrogen limitation there was no induction at all. These results taken together suggest that stationary-phase induction of *mcjA* is triggered by nutrient depletion. The *mcjB* and *mcjC* genes were also regulated by the growth phase of the culture, but in contrast to *mcjA*, they showed substantial expression already during exponential growth. Induction of the microcin genes was demonstrated to be independent of RpoS, the cyclic AMP-Crp complex, OmpR, and H-NS. Instead, we found that the growth-phase-dependent expression of *mcjA*, *mcjB*, and *mcjC* may be explained by the concerted action of the positively acting transition state regulators ppGpp, Lrp, and integration host factor. Measurements of microcin J25 production by strains defective in these global regulators showed a good correlation with the reduced expression of the fusions in such mutant backgrounds.**

Microcin J25 (MccJ25) is a plasmid-encoded, 2,107-Da cyclopeptide antibiotic of 21 unmodified amino acids, produced and excreted into the culture medium by the *Escherichia coli* strain AY25, isolated from human feces (5, 39). As with other microcins but unlike colicins, production of MccJ25 is neither lethal nor stimulated by DNA-damaging treatments that activate the SOS response (3, 38). MccJ25 is primarily active on gram-negative bacteria related to the producer strain, inducing cell filamentation in an SOS-independent way (39). Thus, in addition to its interest as an antibacterial compound, MccJ25 holds promise as a tool for cell division studies. Some pathogenic bacteria, including *Salmonella* and *Shigella* species, are hypersensitive to MccJ25 (39). MccJ25 uptake into *E. coli* is dependent on the cell envelope proteins FhuA, TonB, ExbB (and possibly ExbD), and SbmA (40, 41). We have reported the molecular characterization of the four plasmid genes, *mcjABCD*, involved in microcin synthesis and immunity (45, 46). While active MccJ25 may be extracted from cells expressing the three genes *mcjABC*, no active peptide was detected in cells bearing plasmids that expressed only two genes, *mcjA* and *mcjB* or *mcjA* and *mcjC* (45). Therefore, McjB and McjC must take part in MccJ25 maturation, which would imply the removal of an N-terminal leader of 37 amino acids from the 58-residue precursor McjA, followed by the head-tail cyclization of the 21-residue C-terminal propeptide (5). The microcin immunity protein, McjD, which is highly similar to many ATP-

binding cassette exporters, was found to be required for MccJ25 secretion (45). Thus, the immunity conferred by McjD could be mediated by active efflux of the peptide, which would keep its intracellular concentration below a critical level. Also, we have found that the *E. coli* outer membrane protein TolC may be implicated in the secretion of MccJ25, possibly by forming an export complex with McjD (12).

We previously noted that production of MccJ25 increased when cells reached the stationary phase (39). To understand the molecular basis of this growth-phase-dependent regulation we have constructed fusions between *lacZ* and the genes *mcjA*, *-B*, and *-C*, involved in production of the antibiotic. This report consists of a study of the physiological and genetic factors affecting the expression of these fusions. Our results suggest that the stationary-phase increase is triggered by nutrient depletion. Induction of the microcin genes was shown to be independent of RpoS, the cyclic AMP (cAMP)-cAMP receptor protein (C/RP) complex, OmpR, and H-NS. By examining the effect of mutations in a number of other regulatory loci on expression of *mcjABC* we have found that ppGpp and the proteins Lrp and integration host factor (IHF) are required for the growth phase induction. In addition, the histone-like protein H-NS acts as a positive regulator of *mcjB* but has a negative effect on the expression of *mcjC*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacteria and plasmids used in this study are listed in Table 1. All strains were *E. coli* K-12 derivatives. The *rpoS::Tn10*,  $\Delta relA251::kan$ ,  $\Delta spoT207::cat$ , and  $\Delta himA82-Tn10$  alleles were introduced into MC4100 by P1 transduction (31), using strains ZK1171, RO98, and RO71 as donors. The presence of the *rpoS::Tn10* allele was determined by the reduced oxygen evolution when colonies were flooded with hydrogen perox-

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<b>Strains</b>		
MC4100	<i>araD139</i> $\Delta$ ( <i>argF-lac</i> )205 $\lambda^-$ <i>flbB5301 ptsF25 relA1 rpsL150 deoC1</i>	CGSC <sup>a</sup>
RYC1000	MC4100 $\Delta$ <i>rib-7 recA56 gyrA</i>	F. Moreno
POI1681TR	F <sup>-</sup> Mu dI1681 (Km <sup>r</sup> ) <i>ara::</i> (Mu cts) <sup>3</sup> $\Delta$ ( <i>proAB-argF-lacIPOZYA</i> ) XIII <i>rpsL recA56 srl::Tn10</i> (Tc <sup>r</sup> )	8
ZK1171	W3110 $\Delta$ <i>lacU169 tna-2 rpoS::Tn10</i>	R. Kolter
RO71	MC4100 $\phi$ ( <i>csi-5::lacZ</i> ) ( $\lambda$ <i>placMu55</i> ) $\Delta$ <i>himA82-Tn10</i>	R. Hengge-Aronis
RO64	MC4100 <i>lpp-201::Tn10</i>	R. Hengge-Aronis
RH74	MC4100 $\Delta$ <i>cya851 ilv::Tn10</i>	R. Hengge-Aronis
ZK213	Hfr <i>rpsL</i>	R. Kolter
ZK216	ZK213 $\Delta$ <i>crp45</i>	R. Kolter
RO98	MC4100 $\Delta$ <i>relA251::kan</i> $\Delta$ <i>spoT207::cat</i>	R. Hengge-Aronis
MJ150	MC4100 $\Delta$ <i>himA82-Tn10</i>	This study
MJ152	MC4100 $\Delta$ <i>spoT207::cat</i>	This study
MJ153	MC4100 <i>rpoS::Tn10</i>	This study
RYC514	MC4100 <i>ompR101</i>	F. Moreno
CU284	MC4100 <i>hms::neo</i>	C. Ueguchi
CC118	F <sup>-</sup> $\Delta$ ( <i>ara-leu</i> )7697 <i>araD139</i> $\Delta$ <i>lacX74 galE galK</i> $\Delta$ <i>phoA20 thi rpsE rpoB argE</i> (Am) <i>recA1 appR1</i>	28
CC170	As CC118, with a chromosomal insertion of <i>TnlacZ</i>	28
pop3001.6	MC4100 Mu cts	F. Moreno
<b>Plasmids</b>		
pTUC202	pACYC184 with 6-kb <i>Bam</i> HI- <i>Sal</i> I fragment encompassing <i>mcjABC</i> D genes	45
pTUC341	pBR322 with 5.2-kb <i>Hind</i> III- <i>Sal</i> I fragment encompassing <i>mcjABC</i> D genes	45
pMcjA-LacZ	pTUC202 carrying <i>mcjA::lacZ</i> gene fusion	This study
pMcjB-LacZ	pTUC202 carrying <i>mcjB::lacZ</i> gene fusion	This study
pMcjC-LacZ	pTUC202 carrying <i>mcjC::lacZ</i> gene fusion	This study
pMcjA-LacZ(op)	pTUC202 carrying <i>mcjA::lacZ</i> operon fusion	This study

<sup>a</sup> CGSC, *E. coli* Genetic Stock Center.

ide. The standard Luria-Bertani (LB) broth and M63 minimal medium have been previously described (31). M63 medium was supplemented with glucose (0.2%) and vitamin B<sub>1</sub> (1  $\mu$ g/ml). Solid media contained 1.5% agar. When specified, the media were supplemented with ampicillin (50  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), tetracycline (15  $\mu$ g/ml), chloramphenicol (30  $\mu$ g/ml), or 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) (40  $\mu$ g/ml). All cultures were incubated at 37°C. Growth was monitored by measuring optical density at 600 nm (OD<sub>600</sub>). Aerobic cultures for  $\beta$ -galactosidase assays (50-ml volume in 250-ml Erlenmeyer flasks) were inoculated with overnight cultures grown in the same medium and incubated with vigorous shaking. To ensure the dilution of products from previous induction during stationary phase, cells from overnight cultures were diluted 1:100 in LB broth or M63-glucose and grown to early log phase. Samples were then taken and used to inoculate (1:100) LB or M63 cultures. For anaerobic growth, liquid cultures were grown without agitation in filled tubes with the medium overlaid with mineral oil or in Oxoid anaerobic jars with an H<sub>2</sub>-CO<sub>2</sub> atmosphere generated by using Oxoid gas generating kits.

To make conditioned LB medium, an overnight culture of MC4100(pMcjA-LacZ) was diluted 1:100 into 50 ml of fresh LB broth and left to grow aerobically for 7 h (OD<sub>600</sub>, 2.3) until the same state at which expression of the fusion had been observed to be induced. After growth, the cells were spun down twice and the medium was filter sterilized and used no later than 24 h after it had been prepared. Starvation by depletion of glucose or phosphate was obtained by growing cultures in M63 medium with 0.02% glucose or 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, respectively. In the latter case, 40 mM MOPS (morpholinepropanesulfonic acid; pH 7) was used to provide buffering capacity of the low-phosphate medium. A medium limited in nitrogen was obtained by replacing ammonium sulfate in M63 with an equimolar concentration of potassium sulfate and by adding 1.5 mM ammonium chloride as the sole source of nitrogen.

**Assay of antibiotic activity.** MccJ25 activity in supernatants was determined by the critical dilution method as described previously (39). The microcin titer is reported as the reciprocal of the last dilution which gave a clear spot.

**Isolation and characterization of *lacZ* fusions to microcin genes.** Translational *lacZ* fusions to *mcjABC* were constructed by *TnlacZ* insertion mutagenesis of pTUC202, essentially as described previously (28). Plasmid pTUC202 was transformed into strain CC170, which carries the *TnlacZ* transposable element on the chromosome. Independently mutagenized cultures were plated on LB medium containing 300  $\mu$ g of kanamycin per ml in addition to chloramphenicol to select *TnlacZ* transpositions onto the multicopy plasmid. Resistant colonies were

pooled, and plasmid DNA was extracted and used to transform the  $\Delta$ *lacX74* strain CC118 with selection for Cm<sup>r</sup> Km<sup>r</sup> on LB agar containing X-Gal. Transformants that produced blue colonies were purified, and those that had lost MccJ25 production were retained for further analysis. To identify the gene inactivated by the insertions we did a complementation analysis using strain RYC1000 (*recA*), which was transformed with all possible combinations of pTUC202::*TnlacZ* MccJ25<sup>-</sup> derivatives and a set of pTUC341::Tn5 compatible mutant plasmids with well-characterized insertions in *mcjA*, *mcjB*, or *mcjC* (45). Selection was done on LB medium containing chloramphenicol, kanamycin, and ampicillin. Complementation was considered positive when the two plasmids together restored the MccJ25 production phenotype. Three plasmids bearing fusions to the *mcjA*, *-B*, and *-C* genes, designated pMcjA-LacZ, pMcjB-LacZ, and pMcjC-LacZ, respectively, were selected for further study. The DNA sequences of the fusion joints were determined by the chain termination method (43). Sequencing reactions were primed with a synthetic oligonucleotide primer that hybridized to the N-terminal segment of the *lacZ* gene present in the transposon.

Operon *lacZ* fusions to *mcjA* were obtained by Mu dI1681 insertion mutagenesis (8). Strain POI1681TR(pTUC202) was used to produce transducing particles, which were used to infect pop3001.6. The transduction mixture was incubated at 30°C for 2 h to allow expression of antibiotic resistance and then was plated on LB medium containing kanamycin, chloramphenicol, and X-Gal. Blue colonies were tested for MccJ25 production. Nonproducing plasmid DNAs were isolated, and inactivation of *mcjA* was tested by complementation analysis, as outlined above for *TnlacZ* insertions. The position of the Mu dI1681 element was confirmed by restriction enzyme mapping. To circumvent the problems that Mu dI1681 is transposition proficient and the fusion strain is temperature sensitive for growth, a selected fusion was stabilized by deleting DNA between a *Hind*III site within Mu dI1681 and the unique *Hind*III site in pTUC202 (see Fig. 6A). The resulting plasmid, designated pMcjA-LacZ(op), was retained for further study.

**Other genetic and DNA techniques.** Plasmid DNA minipreps were prepared by using the Wizard kit of Promega. Phage P1 *vir* was used for routine transduction of genetic markers (31). *E. coli* strains were transformed with plasmid DNA by the CaCl<sub>2</sub> procedure (42).

**$\beta$ -Galactosidase assays.**  $\beta$ -Galactosidase activity was measured as described by Miller (31), using cells permeabilized with sodium dodecyl sulfate and chlo-

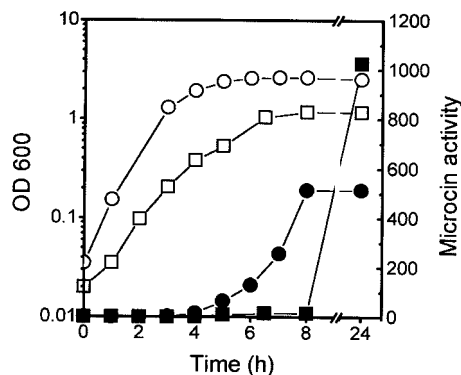


FIG. 1. Growth phase regulation of microcin activity. Strain MC4100(pTUC202) was grown in LB (circles) and M63 (squares) media. The OD<sub>600</sub> (open symbols) and microcin activity (closed symbols) in the two cultures were determined. Microcin activity is expressed as the reciprocal of the last dilution which gave a clear spot (see Materials and Methods).

reform, and is reported in Miller units. The assays were repeated at least twice for each sample.

## RESULTS

**Growth-phase-dependent production of MccJ25.** We have shown previously that MccJ25 production by cells carrying the low-copy-number, natural plasmid pTUC100 is virtually undetectable during early and mid-exponential growth; the antibiotic appears when cells approach the stationary phase (39). The MccJ25 genes from pTUC100 were then cloned into a low-copy-number vector to yield pTUC202, a pACYC184 derivative which carries a 6.2-kb *Bam*HI-*Sal*I fragment containing the *mcjABCD* genes (see Fig. 6A) (45). To examine whether MccJ25 produced from this plasmid showed the same kinetics of synthesis as that produced by the wild-type plasmid, the antibiotic activity of cultures of strain MC4100(pTUC202) in LB medium and M63-glucose minimal medium was measured during both exponential growth and the stationary phase. No assayable or very low amounts of the antibiotic could be detected in supernatants from cells in the exponential phase of growth in both media (Fig. 1). As with the wild-type plasmid, the activity sharply rose in the early stationary phase. Thus, pTUC202 mimics the growth phase regulation of the wild-type system.

**Growth-phase-dependent expression of *mcjA*, *mcjB*, and *mcjC*.** In order to obtain quantitative data on the expression of microcin genes by a convenient assay, we used pTUC202 to construct translational fusions between *mcjA*, *-B*, and *-C* and *lacZ* (as indicated in Materials and Methods). Three representative mutant plasmids, designated pMcjA-LacZ, pMcjB-LacZ, and pMcjC-LacZ, with *TnlacZ* insertions in *mcjA*, *mcjB*, and *mcjC*, respectively, were selected and characterized in detail. The fusion joints from the three plasmids were determined by DNA sequencing, showing that codons 43, 11, and 122 of *mcjA*, *mcjB*, and *mcjC*, respectively, were connected to the '*lacZ*' coding region in proper reading frame. In all three plasmids, *TnlacZ* insertion completely blocked MccJ25 production, as shown by the absence of inhibition zones on a lawn of indicator

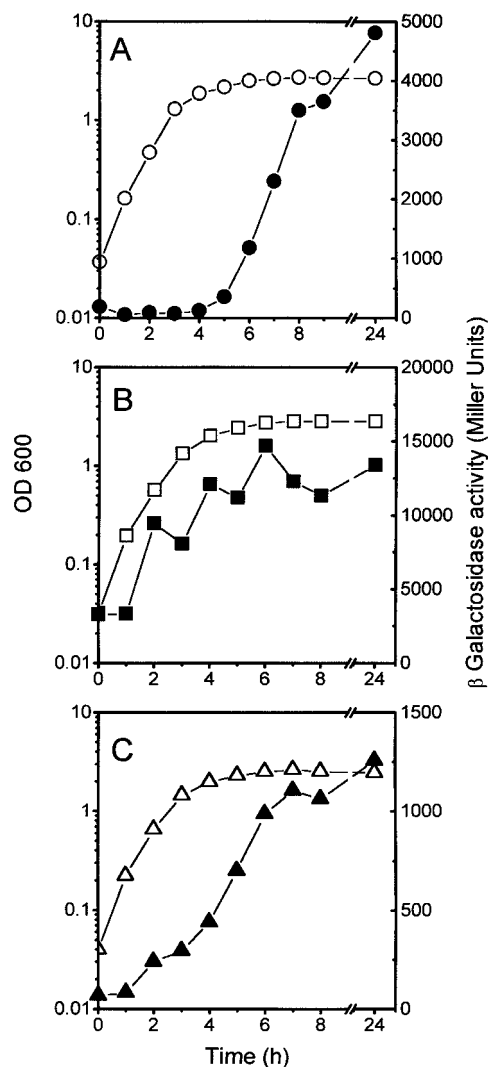


FIG. 2. Growth-phase-dependent expression of MccJ25 genes. Strains MC4100(pMcjA-LacZ), MC4100(pMcjB-LacZ), and MC4100(pMcjC-LacZ) were grown in LB medium. Open and closed symbols show OD<sub>600</sub> and β-galactosidase activities, respectively. (A) Expression of *mcjA::lacZ*; (B) expression of *mcjB::lacZ*; (C) expression of *mcjC::lacZ*.

cells, even when a deferred-antagonism test was employed to increase the sensitivity of detection.

The effect of the growth phase of cells on the expression of *mcjA*, the gene encoding the microcin precursor, was measured with the *mcjA::lacZ* fusion in plasmid pMcjA-LacZ. In LB medium, β-galactosidase activity was low (about 60 U) throughout the exponential phase but began to increase concomitantly with growth transition from exponential to stationary phase (Fig. 2A). Twenty-four hours after inoculation 4,800 U of β-galactosidase was detected, an 80-fold increase with respect to early log phase. Expression of the fusion was similarly induced in the late exponential phase in M63 minimal medium, except that the levels observed in stationary phase (20,000 U in an overnight culture) were higher than those in LB medium. This is consistent with the higher activities of MccJ25 found in supernatants of cultures in minimal medium

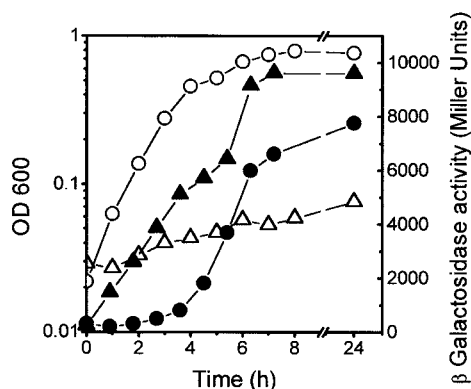


FIG. 3. Expression of the *mcjA::lacZ* fusion in plasmid pMcjA-lacZ in conditioned LB medium and in conditioned LB medium supplemented with fresh medium. MC4100(pMcjA-lacZ) was grown in LB medium that had been conditioned as indicated in Materials and Methods (triangles) and in conditioned LB medium supplemented with 1/15 volume of 5× fresh LB medium (circles). Open and closed symbols show OD<sub>600</sub> and β-galactosidase activities, respectively.

of MC4100 harboring plasmid pTUC100, compared with those in LB medium (39). As shown in Fig. 2B and C, *mcjB* and *mcjC* are also regulated by the growth phase of the culture. However, in contrast to *mcjA*, there was substantial expression from these genes during exponential growth. The *mcjC::lacZ* fusion expresses relatively low levels of β-galactosidase compared to *mcjB::lacZ*. Although *mcjB* and *mcjC* have been proposed to form an operon (46), we suspect that the concentration of McjC in the cells may be low, possibly due to a reduced translation efficiency. This presumption would be supported by our repeated failure to visualize the McjC polypeptide in maxicells, while McjB was readily identified (J. O. Solbiati, R. N. Fariás, and R. A. Salomón, unpublished data).

#### Influence of environmental factors on *mcjA::lacZ* expression.

Various factors could be responsible for the enhanced expression of *mcjA* in stationary phase, including the accumulation of compounds excreted by the cells, nutrient depletion, pH changes, reduced oxygen availability, or high cell density. The following experiments addressed the identity of the factor(s) responsible for triggering the induction at the transition from exponential to stationary phase.

(i) ***mcjA::lacZ* expression in conditioned LB.** We studied the induction kinetics of *mcjA::lacZ* in LB medium that had been used for cell growth (conditioned LB medium [see Materials and Methods]). Exponentially growing cells of strain MC4100 (pMcjA-LacZ) were suspended in culture supernatant recovered from early-stationary-phase cells. *mcjA* was induced immediately in cells exposed to spent medium (Fig. 3), while in fresh LB medium induction did not start until the onset of stationary phase (Fig. 2A). To test whether this behavior was dependent on either accumulation or depletion of a compound, 1/15 volume of 5× fresh LB medium was added to the conditioned medium (addition of concentrated fresh medium avoided excessive dilution of a putative inducer). As observed in fresh medium, induction occurred in the supplemented medium only upon the onset of the stationary phase (Fig. 3). Thus, the stationary-phase induction was not due to an inducer released by the cells used to deplete the medium.

(ii) **Effect of oxygen limitation.** The fact that induction in conditioned medium was observed in cells growing at low density in the presence of oxygen suggested that a factor(s) other than a decrease in the oxygen partial pressure or cell density was responsible for the effect. We tested the effect of anoxia on the increase in β-galactosidase activity from *mcjA::lacZ*. It was found that anaerobic conditions imposed during early exponential phase reduced expression of the fusion. Late-stationary-phase cells from anaerobic cultures contained β-galactosidase levels sevenfold lower than those of stationary-phase cells from aerobic cultures (700 versus 4,800 U). This is consistent with the observation that production of microcin is greater at high aeration (data not shown).

(iii) **Effect of pH.** The pH of the LB medium when the experiments were started was 7, but cultures grown in LB medium become alkaline in stationary phase. To test whether this increase in pH was responsible for the induction of MccJ25 expression, 40 mM MOPS was added to buffer the LB medium. In another experiment, the β-galactosidase activity of the *mcjA::lacZ* fusion was tested in fresh LB medium adjusted to pH 8 with NaOH before inoculation. Cultures grown in these media showed induction kinetics and levels of expression comparable to those of controls (data not shown), demonstrating that an increase in pH in stationary-phase cultures did not affect *mcjA* expression.

(iv) **Effect of carbon, phosphorus, and nitrogen limitations.** Limitation for some nutrient might be the signal for increasing microcin levels late in exponential phase. To determine whether the fusion was induced upon starvation for different macronutrients, MC4100 (pMcjA-LacZ) was grown in M63 medium which was limited for either glucose, phosphate, or ammonia (see Materials and Methods). A halt of the growth provoked by a 10-fold reduction in glucose availability was followed by an increase in β-galactosidase activity (Fig. 4A). Yet the induction kinetics was slower and the absolute level of expression was reduced compared with those observed in standard M63-glucose medium. Phosphate depletion strongly stimulated *mcjA* expression (Fig. 4B). In contrast, no induction was seen under conditions of ammonia starvation (Fig. 4C).

**Stationary-phase induction of *mcjA* expression does not require *rpoS*, *cya*, *crp*, *ompR*, or *hns* function.** The increase in *mcjA* expression as cell growth slows, especially during growth in rich medium, is characteristic of genes regulated by the *rpoS*-encoded sigma factor  $\sigma^S$  (27). We measured β-galactosidase activity at different growth phases with ZK1171 (*rpoS::Tn10*) cells harboring plasmid pMcjA-LacZ. The mutation decreased late-stationary-phase levels of β-galactosidase by 40%, but an induction ratio of 60 was still observed, indicating that the growth phase response of the *mcjA* gene does not require the presence of an intact *rpoS* gene, thus resembling many stationary-phase-induced genes which are independent of  $\sigma^S$  regulation (21, 34).

It is well known that the growth-dependent expression of many genes requires cAMP and the cAMP receptor protein (CRP) (44). We therefore tested the expression in LB medium of *mcjA::lacZ* in *cya* (strain RH74) and *crp* (strain ZK216) mutants, along with their isogenic parents MC4100 and ZK213, respectively. The final β-galactosidase levels in the *cya* and *crp* strains were reproducibly decreased by 20 and 43% with respect to those observed for the wild-type controls. How-

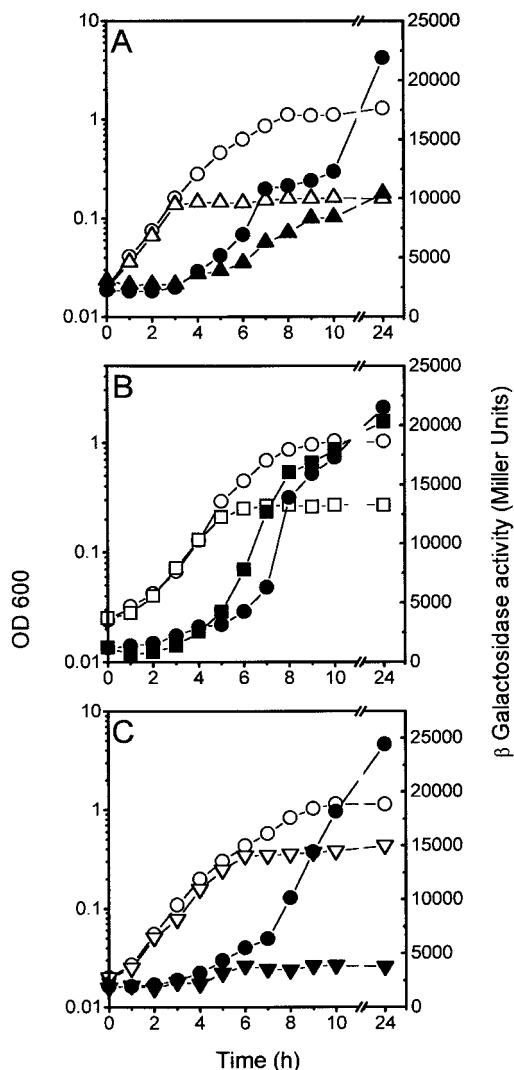


FIG. 4. Effects of starvation for carbon, phosphorus, or nitrogen on the expression of *mcjA::lacZ*. Strain MC4100(pM*cjA*-LacZ) was grown in minimal M63 medium with limiting concentrations of glucose (A) (triangles), phosphate (B) (squares), or ammonium (C) (inverted triangles). The control grown in standard M63 medium (circles) is included in all three panels. At the time intervals indicated, OD<sub>600</sub> (open symbols) and  $\beta$ -galactosidase activities (filled symbols) were measured.

ever, the mutant strains still showed the induction in stationary phase. It is interesting that microcin levels in stationary-phase cultures of the natural producer *E. coli* AY25 were twofold higher on glycerol or lactose than on glucose, suggesting carbon catabolite repression of microcin synthesis (39).

It has been shown that stationary-phase levels of MccB17 are greatly enhanced by the regulatory protein OmpR (6, 22), which also controls expression of the outer membrane porins OmpC and OmpF (37). This appears not to be the case for MccJ25, since synthesis of  $\beta$ -galactosidase from the *mcjA::lacZ* fusion in *ompR101* cells (strain RYC514) was found not to be significantly affected (data not shown).

H-NS is a global modulator of gene expression affecting the synthesis, both positively and negatively, of more than 50 *E.*

*coli* proteins (26). Both exponential- and stationary-phase levels of expression from *mcjA::lacZ* were reduced almost threefold in the *hns* mutant CU284 (1,700 versus 4,800 U for MC4100, in overnight cultures), yet the fusion still showed at least 60-fold induction.

**Mutations in *himA*, *lrp*, and *spoT* affect *mcjA* induction.** IHF is a heterodimer made up of two subunits, IHF $\alpha$  and IHF $\beta$ , encoded by the genes *himA* and *hip* (or *himD*), respectively (15). IHF has a direct positive or negative role in the expression of a number of genes in *E. coli* (20). As shown in Fig. 5A, the *himA* mutation in strain MJ150 drastically reduced the  $\beta$ -galactosidase levels from *mcjA::lacZ*, indicating that IHF positively affects the expression of *mcjA*. In support of this observation we have detected several candidate IHF-binding sites in the putative promoter region of the *mcjA* gene (Fig. 6B) that have high identity with the 13-bp consensus sequence WATCAANNNTTR (W = A or T; R = A or G) (10).

The leucine-responsive regulatory protein (Lrp) can be either an activator or repressor of many genes in *E. coli* (33). To test the effect of Lrp on the expression of *mcjA* we transformed RO64 (*lrp201::Tn10*) with plasmid pM*cjA*-LacZ and examined the kinetics of *mcjA-lacZ* expression by measuring  $\beta$ -galactosidase activity in cells grown in LB medium. The *lrp* mutation decreased the expression of *mcjA* to an extent comparable to that of the *himA* mutant (Fig. 5B), indicating that *mcjA* is also under positive control of Lrp. A potential Lrp target consensus sequence was detected upstream from the presumptive *mcjA* promoter region (Fig. 6B). This 12-bp sequence deviates by only one nucleotide from the consensus (TTTATTCTNaAT, where the less-conserved nucleotides are in lower case) defined by Rex et al. (36).

After entry into stationary phase, the level of (p)ppGpp is known to increase (7). Intracellular concentrations of (p)ppGpp are controlled by the *relA* gene, encoding the ribosome-dependent (p)ppGpp synthetase I (or stringent factor), and the *spoT* gene, encoding the ribosome-independent (p)ppGpp 3'-pyrophosphohydrolase-(p)ppGpp synthetase II. The RelA enzyme is required for the rapid increase in (p)ppGpp synthesis following amino acid starvation. The bifunctional SpoT enzyme is required for maintenance of basal levels of (p)ppGpp and is responsible for the RelA-independent (p)ppGpp accumulation following nutrient limitations that do not involve amino acid starvation, such as carbon and energy source exhaustion (7, 17). To examine the effect of (p)ppGpp deficiency on *mcjA* expression, we transformed pM*cjA*-LacZ into strain MJ152 (*relA1 ΔspoT*) and measured  $\beta$ -galactosidase activity in LB medium (Fig. 5C). No induction of *mcjA::lacZ* was seen, indicating that *mcjA* expression is positively regulated by (p)ppGpp. Strain MC4100, which has been employed throughout the present study as a host for fusion plasmids, harbors a *relA1* mutation (30). However, the activity of the *mcjA::lacZ* fusion was very similar in strain W3110, which carries the wild-type *relA* allele (data not shown). Also, no significant difference in  $\beta$ -galactosidase activity from *mcjA::lacZ* was noted when the *relA*-null allele from strain RO98 was substituted for the *relA1* mutation of MC4100. Altogether, these data indicate that induction of *mcjA* is dependent on *spoT* but is *relA* independent. The *relA1* allele apparently has a weak residual (p)ppGpp synthetic activity, which could lead to low basal levels of (p)ppGpp when the nucleotide degradation is severely com-

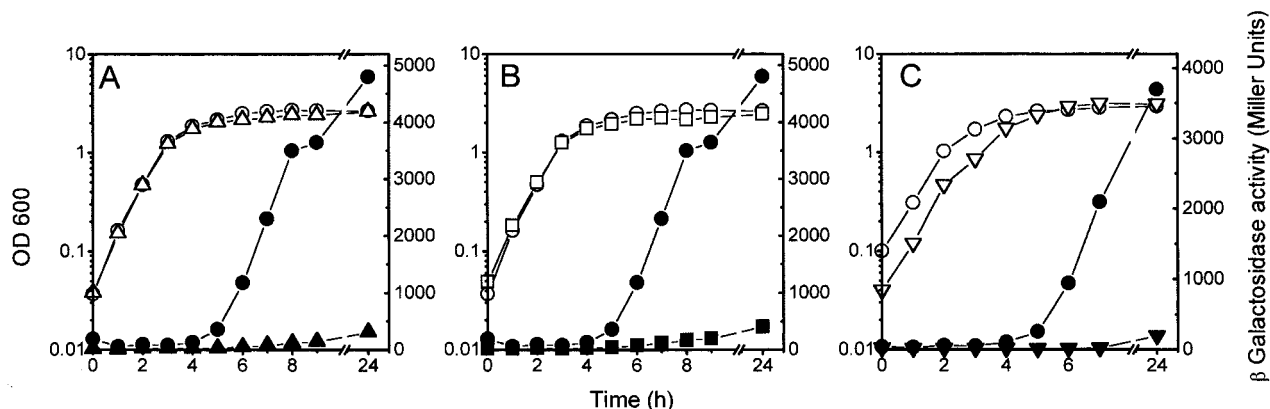


FIG. 5. Effect of *lrp*, *himA*, and *spoT* mutations on growth-phase-dependent *mcjA::lacZ* expression. Strain MC4100(pM<sub>cjA</sub>-LacZ) (circles) and its *himA* (triangles) (A), *lrp* (squares) (B), and *spoT* (inverted triangles) (C) mutant derivatives were grown in LB medium. Optical densities (open symbols) and  $\beta$ -galactosidase activities (closed symbols) were determined along the growth curves.

promised, as in *relA1*  $\Delta$ *spoT* mutants (30). We could not examine the effect of a complete (p)ppGpp depletion on the expression of the fusion, since the available double mutant RO98 ( $\Delta$ *relA251::kan*  $\Delta$ *spoT207::cat*) was already resistant to kanamycin and chloramphenicol, making it impossible to select for acquisition of the fusion plasmid, which has the same antibiotic markers. Instead, we transduced both null mutations to MC4100 harboring pTUC341 (Ap<sup>r</sup>), a pBR322 derivative carrying the MccJ25 genetic system, and measured MccJ25 production. No antibiotic activity was detected in the supernatant of an overnight culture of the double mutant grown in LB medium, while the control culture showed a titer of 512.

**Regulation of expression of a *mcjA::lacZ* operon fusion.** The activity of the *mcjA::lacZ* gene fusion did not permit a distinction between transcriptional or translational controls. In all cases studied so far, IHF, Lrp, and (p)ppGpp function as transcriptional regulators, and it is to be expected that they play a similar role in the expression of *mcjA*. To verify whether the expression of *mcjA* is regulated transcriptionally, we constructed the plasmid pM<sub>cjA</sub>-LacZ(op), harboring an *mcjA::lacZ* transcriptional fusion, as described in Materials and Methods. This plasmid was transformed into strain MC4100 and its mutant derivatives MJ150 (*himA*), RO64 (*lrp*), and MJ152 (*spoT*). As shown in Table 2,  $\beta$ -galactosidase activity from the transcriptional fusion of parent cells increased at the end of the exponential phase but no induction was seen in the mutant cells. Thus, the expression pattern of the transcriptional fusion was similar to that of the translational one, indicating that regulation of *mcjA* is under transcriptional control. However, note that the translational fusion showed lower levels of  $\beta$ -galactosidase and a higher factor of activation than the transcriptional fusion. This difference could possibly be explained by a low stability of the fusion protein. Alternatively, *mcjA* expression may also be subjected to posttranscriptional control.

**Regulation of expression of the *mcjB* and *mcjC* genes.** To determine the effect of global regulators on expression of *mcjBC*, the  $\beta$ -galactosidase activity of *mcjB::lacZ* and *mcjC::lacZ* translational fusions was assayed in the same mutant hosts used for *mcjA*. We found that *cya*, *crp*, *rpoS*, and *ompR* mutants

showed the growth phase increase in expression of these genes (data not shown). Similar to what had been found for *mcjA*, the final  $\beta$ -galactosidase levels of *mcjB::lacZ* in the *cya* strain was decreased by 53% with respect to that observed for the wild type. Also, late-stationary-phase levels of  $\beta$ -galactosidase for *mcjB::lacZ* and *mcjC::lacZ* in the *rpoS* mutant were reduced by 40 and 50%, respectively, with respect to the control. The most remarkable effects were seen with the Lrp-, IHF-, (p)ppGpp-, and H-NS-defective strains. As can be seen in Fig. 7A and C, in the absence of Lrp or IHF, the fusions showed a residual level of expression which was still subject to growth phase regulation. (p)ppGpp deficiency, on the other hand, had a more severe effect, since expression of *mcjB::lacZ* and *mcjC::lacZ* was low and constant throughout the growth cycle. This indicates that (p)ppGpp is the principal factor modulating the expression of *mcjBC* genes and that Lrp and IHF are important for maximum levels of expression from *mcjBC* during stationary phase. To test for H-NS-dependent effects on *mcjB::lacZ* and *mcjC::lacZ*,  $\beta$ -galactosidase activity from the fusions was measured in the isogenic strains MC4100 and CU284 (*hns*). While stationary-phase expression of *mcjB* was reduced threefold (Fig. 7B), we consistently detected a threefold stimulation of the induced level of expression from *mcjC* in the *hns* mutant (Fig. 7D). Thus, these genes were affected in an opposite way by the same regulator.

**The reduced activity of microcin genes in mutant backgrounds is paralleled by a decrease in MccJ25 production.** To verify in a different manner the reduced expression of fusions to microcin genes in *lrp*, *himA*, and *spoT* mutant backgrounds, we measured the effect of these mutations on the production of biologically active MccJ25. Microcin titers in overnight LB cultures of RO64 (*lrp*), and MJ150 (*himA*) harboring plasmid pTUC202 were 256- and 512-fold lower, respectively, than that of the control (titer, 512). No activity was detected in supernatants of MJ152 (*relA1* $\Delta$ *spoT*) transformed with pTUC202.

## DISCUSSION

In this study, the environmental and genetic factors governing the expression of MccJ25 genes were analyzed. We dem-

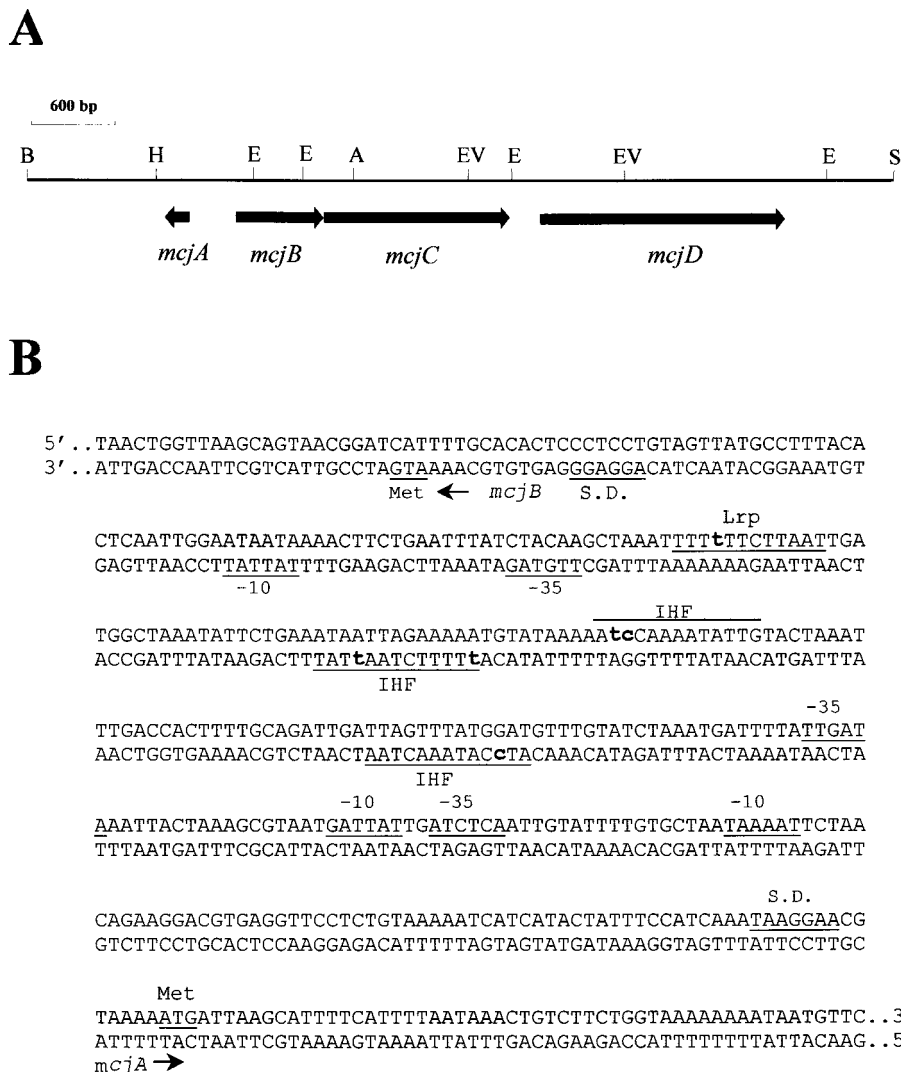


FIG. 6. (A) Physical and genetic map of the DNA fragment (6.2 kb) cloned in pTUC202. Horizontal arrows indicate the *mcjABCD* genes and the direction of transcription. (B) Nucleotide sequence of the *mcjA* and *mcjB* promoter regions, including the beginning of the genes. Note that *mcjA* and *mcjB* are transcribed from the opposite DNA strands (46). Predicted ribosome binding sites (S.D.) and promoter sequences as well as start codons for McjA and McjB translation are shown. Note that two tentative promoter regions (-35 and -10) are shown for *mcjA*. The regions corresponding to possible IHF and Lrp recognition sequences are indicated. Boldface lowercase letters represent the nucleotides which are different from the canonical ones. The sequence of the entire MccJ25 operon has been deposited in the GenBank database under accession no. AF061787.

onstrated that expression of *mcjA*, the gene encoding the MccJ25 precursor, is induced upon transition from exponential to stationary phase in both LB and M63-glucose media. This increase is not dependent on cell density, pH changes, anaerobiosis (which in fact was found to repress the *mcjA* gene), or the buildup of some inducer, suggesting that the stationary-phase induction may result from the depletion of one or more nutrients. In fact, we have shown that carbon and inorganic phosphate starvation were able to promote the growth phase response, while no induction occurred under nitrogen limitation. By using strains deficient in the *E. coli* regulatory proteins RpoS, cAMP-CRP, OmpR, and H-NS, we demonstrated that these regulators are not involved in the growth-phase-dependent expression of *mcjA*.

The principal finding of the present study is that expression of *mcjA* is positively regulated by a complex network of global regulators, including at least Lrp, IHF, and (p)ppGpp. Taking into account that these regulators themselves are growth phase responsive, we propose a model describing their interplay in the regulation of *mcjA* expression. During exponential phase in LB medium the low level of *mcjA* expression may be explained by the low concentrations of (p)ppGpp, Lrp, and IHF (7, 14, 21, 25). In the decelerating phase, the intracellular concentration of (p)ppGpp would start to increase via the SpoT mechanism, which specifically responds to carbon and energy source deprivation. On the other hand, it has been shown that Lrp and IHF levels also display a maximum at the phase transition of cell growth (2, 14, 25). The concerted action of (p)ppGpp, Lrp,

TABLE 2. Effects of *lrp*, *himA*, and *spoT* mutations on expression of an *mcjA::lacZ* transcriptional fusion

Strain <sup>a</sup>	Relevant genotype	β-Galactosidase activity (Miller units) during:		
		Early exponential phase <sup>b</sup>	Late exponential phase <sup>c</sup>	Stationary phase <sup>d</sup>
MC4100	Wild type	1,100	24,300	28,000
RO64	<i>lrp::Tn10</i>	811	1,390	2,595
MJ150	<i>ΔhimA</i>	506	820	1,066
MJ152	<i>relA ΔspoT</i>	956	1,012	1,650

<sup>a</sup> All strains harbor the pM<sub>cjA</sub>-LacZ(op) plasmid.

<sup>b</sup> OD<sub>600</sub>, approximately 0.3.

<sup>c</sup> OD<sub>600</sub>, approximately 2.

<sup>d</sup> OD<sub>600</sub>, approximately 3.

and IHF would stimulate the expression of *mcjA* at the onset of stationary phase. Since (p)ppGpp is required for substantial Lrp and IHF expression (1, 25), it is possible that (p)ppGpp deficiency acts not only directly but also indirectly via its role in the control of Lrp and IHF. If this were so, (p)ppGpp would be the main positive effector of *mcjA* expression. If optimal induction of *mcjA* resulted from the additive effects of the three regulators, one should expect that in the absence of one of them a certain level of induction would remain, promoted by the presence of the other two. However, *mcjA* induction is practically abolished in strains deficient in either (p)ppGpp, Lrp, or IHF (Fig. 5). It seems, therefore, that induction requires the combined action of these factors. Basically, the same

considerations may be invoked to explain the increased activity of the *mcjB* and *mcjC* genes upon entering stationary phase, since they are also positively modulated by IHF, Lrp, and ppGpp.

At present, we cannot decide whether Lrp and IHF directly bind to the *mcjA* regulatory region. However, this seems highly likely since potential binding sites with strong homology to the consensus can be found (Fig. 6B). IHF has been found to directly stimulate transcription from the Pe promoter of bacteriophage Mu (19, 23), the pL1 promoter of bacteriophage lambda (18), and the P<sub>G2</sub> promoter of the *ilvGMEDA* operon of *E. coli* (35). In all three cases, IHF binds to a site located just upstream from the promoter. Note that the putative Lrp- and IHF-binding sites indicated in Fig. 6B not only are close to the *mcjA* promoter region but are also in close proximity to the *mcjBC* promoter region. Therefore, these sites might also participate in the control of the two latter genes.

Another interesting finding of this work is that the histone-like protein H-NS exerts a negative effect on *mcjC* expression, while *mcjB*, in contrast, appears to be positively regulated by this factor. H-NS influences the transcription of a number of genes and, in nearly all cases studied so far, it inhibits their expression. Since *mcjB* and *mcjC* are closely linked (in fact, the end of *mcjB* overlaps the beginning of *mcjC*) (46), it is likely that both genes are directed by the presumptive promoter we have located upstream of *mcjB* (Fig. 6B). It is difficult to imagine how an alteration in the DNA topology mediated by

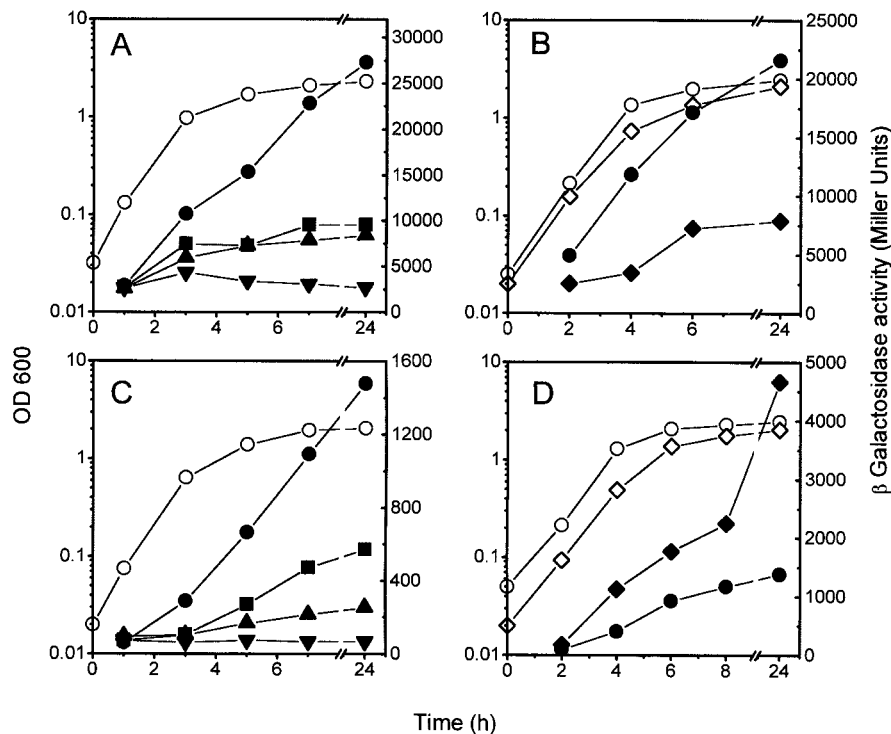


FIG. 7. Effect of global regulators on growth-phase-dependent expression of *mcjB::lacZ* and *mcjC::lacZ* fusions. β-Galactosidase activity (solid symbols) and cell growth (open symbols) were measured in LB medium at the indicated times. (A) MC4100(pM<sub>cjB</sub>-LacZ) (circles) and its isogenic *lrp* (squares), *himA* (triangles), and *spoT* (inverted triangles) mutants; (B) MC4100(pM<sub>cjB</sub>-LacZ) (circles) and its *hns* mutant (diamonds); (C) MC4100(pM<sub>cjC</sub>-lacZ) and its isogenic *lrp* (squares), *himA* (triangles), and *spoT* (inverted triangles) mutants; (D) MC4100(pM<sub>cjC</sub>-lacZ) (circles) and its *hns* mutant (diamonds). For clarity, only the growth curve of the control strain has been represented in panels A and C, as those of the mutants were nearly identical.



the regulator could differentially affect the expression of two genes expressed from the same promoter (we could not find any putative *mcjC* promoter within the coding region of *mcjB*). The possibility of a posttranscriptional control of *mcjC* by H-NS should be considered. In fact, Yamashino et al. (47) and Barth et al. (4) showed that H-NS inhibits the expression of *rpoS* during the exponential phase by a mechanism acting at the posttranscriptional level and that relief of repression by H-NS plays a role in *rpoS* induction upon entry into stationary phase. In light of these findings, we propose that H-NS could also repress *mcjC* and that the higher levels of *mcjC* in *hns* mutants may result from an elevated translational efficiency of *mcjC* mRNA. Clearly, the function of H-NS in the regulation of *mcjBC* warrants further study.

It is worth noting that a correlation between the activity of fusions to microcin genes and MccJ25 synthesis was demonstrated by measuring the production of biologically active MccJ25 in stationary-phase supernatants of cultures of Lrp-, IHF-, and (p)ppGpp-deficient strains. In all cases, the reduced expression of *mcjABC* in the mutant backgrounds was paralleled by a corresponding decrease in MccJ25 activity.

Like most antibiotics, synthesis of microcins is induced when cultures cease exponential growth (3, 29). The regulatory mechanisms underlying the production of microcins have been studied in detail only for MccB17 and MccC7. Expression of the MccB17 operon is induced when cells stop growing because of exhaustion of glucose, ammonia, or phosphate and also when cells enter stationary phase in rich medium (9). Stationary-phase induction of MccB17 does not require  $\sigma^s$  (6), cAMP (9), or (p)ppGpp (9). The expression of MccB17, on the other hand, is activated by OmpR (6, 22) and IHF (32), while Emr (also known as MprA) negatively regulates transcription from the major promoter of the operon (11). Expression of MccC7 appears to be activated not by OmpR and IHF but by the products of the chromosomal genes *crp* and *appR* (13, 32). The expression of colicins E1 and K is also growth phase dependent. Eraso et al. (16) demonstrated that expression of the colicin E1 structural gene, *cea*, is stimulated when cells reach the stationary phase. This increase in expression is due to depletion of nutrients and was found to be independent of the SOS response, anaerobiosis, and catabolite repression as well as the regulators IHF, RpoS, and Lrp. Nutrient depletion also induces the expression of the colicin K structural gene *cka* through an increase in ppGpp (24). This induction is independent of the cAMP-CRP complex, RpoS, Lrp, and H-NS. From a comparison of the regulatory patterns of these colicins and microcins with that of MccJ25 several conclusions can be drawn. First, nutrient depletion is the common signal for stationary-phase induction of all of them. Second, neither RpoS nor the cAMP-CRP complex is involved in the growth-phase response (except for MccC7, whose production is undetectable in *cya* or *crp* strains). Third, although these compounds share some regulatory factors, induction of individual microcins and colicins is further influenced by specific regulators.

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