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Protective Action of ppGpp in Microcin J25-Sensitive Strains▽

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As *Escherichia coli* strains enter the stationary phase of growth they become more resistant to the peptide antibiotic microcin J25. It is known that starvation for nutrients such as amino acids or glucose leads to increases in guanosine 3′,5′-bispyrophosphate (ppGpp) levels and that the intracellular concentration of this nucleotide increases as cells enter the stationary phase of growth. Therefore, we examined the effects of artificially manipulating the ppGpp levels on sensitivity to microcin J25. A direct correlation was found between ppGpp accumulation and microcin resistance. Our results indicate that the nucleotide is required to induce production of YojI, a chromosomally encoded efflux pump which, in turn, expels microcin from cells. This would maintain the intracellular level of the antibiotic below a toxic level.

Microcin J25 (MccJ25) is a plasmid-encoded, 21-amino-acid, antibacterial peptide produced by *Escherichia coli* (32). It is active against *E. coli* and *Salmonella* and *Shigella* spp. Production of MccJ25 largely occurs as cultures enter stationary phase, and this timing is regulated by the concerted action of the positively acting transition state regulators guanosine 3′,5′-bispyrophosphate (ppGpp), leucine-responsive regulatory protein (Lrp), and integration host factor (10). MccJ25 is primarily active on gram-negative bacteria related to the producer strain, inducing cell filamentation in an SOS-independent way; some pathogenic bacteria, including *Salmonella* and *Shigella* species, are hypersensitive to MccJ25 (32). Four genes (*mcjA*, *mcjB*, *mcjC*, and *mcjD*) are required for MccJ25 synthesis, export, and immunity (38, 39). *mcjA* encodes the primary structure of MccJ25 as a 58-amino-acid precursor, from which an N-terminal leader of 37 amino acids is removed. The 21-residue mature peptide has a compact, extraordinary structure, which consists of an 8-residue lariat ring and a C-terminal tail that folds on itself and passes through the ring, where it is sterically trapped (4, 31, 47). The *mcjB* and *mcjC* gene products are involved in this process (11, 16). The *mcjD* product has a dual role. It works as a dedicated exporter of MccJ25 and, at the same time, by ensuring rapid secretion from the cytoplasm, protects cells from endogenous MccJ25 synthesized in producer cells, as well as from the exogenous microcin that gains entry (14, 39).

Entry of microcin in target cells is mediated by the outer-membrane receptor FhuA and the inner-membrane proteins TonB, ExbB, ExbD, and SbmA (33, 34). *E. coli* RNA polymerase (RNAP) is the target of antibiotic action (13, 48). The binding site for MccJ25 is located in the secondary channel of the enzyme (48), which provides a route by which the nucleotide substrates reach the catalytic site. Thus, MccJ25 would inhibit transcription by clogging the conduit, thereby blocking the access of the substrates to the active center (1, 25). Recently, we have found that in *E. coli* cells overproducing the microcin receptor, FhuA, the antibiotic also targets the respiratory chain and inhibits cell respiration (5). Although the mechanistic details of this action have yet to be defined, it seems to result from an increased superoxide production. These results indicate that MccJ25 has at least two different intracellular targets.

When enterobacteria such as *E. coli* are starved for amino acids, they elicit the stringent response, characterized by the accumulation of the bacterial alarmones, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (9), collectively referred to as ppGpp. The levels of ppGpp have been found to be inversely correlated with growth rates (9) and to affect the expression of traits important to the virulence of many different bacteria, including biofilm formation (3, 42), quorum sensing and competence development (19, 44), antibiotic synthesis (40), and bacteriocin production (21). In *E. coli* and related gram-negative enteric bacteria the intracellular concentrations of ppGpp are controlled by the *relA* gene, encoding the ribosome-dependent ppGpp synthetase I (or stringent factor), and the *spoT* gene, encoding bifunctional ppGpp synthetase II/3′-pyrophosphohydrolase (9). Inactivation of *relA* and *spoT* leads to a ppGpp-null phenotype, ppGpp^o. Alterations in the intracellular level of ppGpp have pleiotropic effects on metabolism. The nucleotide binds to the β and β′ subunits of the RNA polymerase core enzyme (2), modifying polymerase specificity, and affects a plethora of physiological activities, the main target being transcription. It represses rRNA and protein synthesis (41), stimulates the metabolism of certain amino acids (7), and can also act as a positive effector of gene expression, and a large number of genes require this nucleotide for their induction during starvation (22).

The starting point of the present study was the observation that *E. coli* cells taken from an LB culture within 2 h after entry in stationary phase proved less sensitive to MccJ25 compared to log-phase cells. This led us to hypothesize that the increased

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TABLE 1. Bacteria and plasmids used

Strain or plasmid	Genotype or description	Source or reference
Strains		
CF4941	MG1655 *galK2 zib563*::Tn*10* Δ*relA251*	M. Cashel
CF4977	CF4941 *spoT202*	M. Cashel
CF4943	CF4941 *spoT203*	M. Cashel
MC4100	F$^-$ *araD139* Δ(*argF-lac*)205λ$^-$ *rpsL150 flbB5301 relA1 deoC1 pstF25*	CGSC[a]
SBG231	AB259 spontaneous MccJ25^r mutant (*rpoC* T931I)	13
AB1133	F$^-$ *thr-1 ara-14 leuB6 lacY1* Δ(*gpt-proA*)*62 supE44 galK2* λ$^-$ *rac hisG4 rfbD1 rpsL31* (Sm^r) *kdgK51 xyl-5 mtl-1 argE3 thi-1*	CGSC
RO98	MC4100 Δ*relA251*::*kan* Δ*spoT207*::*cat*	R. Hengge-Aronis
SBS100	MC4100 *yojI*::*lacZ*	S. B. Socías
Plasmids		
pCNB0209R	P_{lac}-His-*relA*	8
pALS13	P_{tac}-*relA′* (RelA 1-455); Ap^r	41
pPAV01	pACYC184 carrying *fhuA*	P. A. Vincent
pFP348	pACYC184 carrying *mcjABC*	This study

[a] CGSC, *E. coli* Genetic Stock Center.

levels of ppGpp in stationary phase might play a role in this phenotype. To test this possibility, the influence of ppGpp was analyzed with *E. coli* mutant strains with a partial or complete ppGpp deficiency and by using recombinant plasmids carrying the *relA* gene that direct the overproduction of ppGpp. We show here that indeed there is a dependence of resistance to exogenous MccJ25 on ppGpp concentration. Furthermore, we demonstrate that this phenotype results from a stimulation by ppGpp of MccJ25 export by the inner-membrane protein YojI.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains and plasmids used in the present study are listed in Table 1. Cultures were grown with shaking at 37°C in Luria broth (LB). Growth was monitored by measuring the optical density at 600 nm (OD_{600}). M9 minimal medium was supplemented with glucose (0.2%) and thiamine (1 mg/ml). Solid media contained 1.5% agar. Antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; and kanamycin, 30 μg/ml.

General methods. Plasmid DNA was isolated with the Wizard Miniprep DNA purification system (Promega) according to manufacturer's instructions. Transformation of competent cells by the $CaCl_2$ procedure was done as described previously (35). Transductions were performed with bacteriophage P1*vir* by the method of Miller (24).

Sensitivity test and bioassay of microcin activity. MccJ25 was purified as previously described (6). For a reliable comparison of the sensitivities of different strains, we used a spot-on-lawn test for determining the MIC of MccJ25 for each strain, as follows. Doubling dilutions of the pure microcin preparation (1 mg/ml) were spotted (10 μl) onto LB plates and dried. Aliquots (50 μl) of cultures to be tested for sensitivity, in stationary phase, were mixed with 3 ml of top agar (0.7% agar) and overlaid onto the plates. After overnight incubation, the plates were examined for different degrees of inhibition; the higher the last dilution which produced a spot, the more sensitive the strain tested.

To test a strain's ability to produce extracellular MccJ25, it was grown on solid LB medium, and a fresh colony was stabbed with a toothpick into the assay plate, which contained M9 medium. After 24 h of growth at 37°C, cells were killed with chloroform. The plates were then overlaid with approximately 10^8 sensitive indicator cells in 3 ml of soft agar. The presence of excreted microcin was indicated by a zone of growth inhibition in the lawn of indicator cells surrounding the stabbed colony.

Construction of a *yojI-lacZ* transcriptional fusion. We started from a derivative of DH5α strain, in which the *yojI* gene had been replaced by a chloramphenicol resistance cassette via a λ Red recombinase-mediated gene replacement (15). The Δ*yojI* mutation was then transduced into strain MC4100, and the cassette was subsequently removed by using the FLP recombinase produced by a conditionally replicating plasmid (12), thus creating an unmarked *yojI* deletion. The single-copy *lacZ* transcriptional fusion to *yojI* was constructed on MC4100 Δ*yojI* using plasmid pKG136, as described previously (17). The *yojI*::*lacZ* fusion strain was designated SBS100.

Effect of MccJ25 on the in vivo incorporation of labeled uridine on RNAs. Strains AB1133 and AB1133(pALS13) were grown in M9-glucose to early exponential phase (OD_{600} = 0.2 to 0.3), and the culture was split into two parts. One of them received MccJ25 at a final concentration of 0.8 μM, and the other served as a control. After a 15-min preincubation, 75 μg of uridine per ml and 3 μCi of [^{3}H]uridine were added to the cells. At the times indicated, 0.5-ml portions were removed from the flasks, mixed with 1.5 ml of 5% cold trichloroacetic acid, and chilled on ice for 1 h. Each sample was filtered through a Millipore HAWP02500 filter and washed with 10 ml of cold trichloroacetic acid. The radioactivity retained on the dried filters was estimated in a Beckman LS-1801 liquid scintillation counter. Triplicate samples were taken at each time point.

β-Galactosidase assay. Strains SBS100 (carrying a *yojI*::*lacZ* transcriptional fusion) and SBS100(pALS13) were grown in LB with or without 0.5 μM MccJ25 to exponential phase. The β-galactosidase activity was measured as described by Miller (24), using cells permeabilized with sodium dodecyl sulfate and chloroform, and is reported in Miller units. The assays were repeated at least five times for each sample.

Oxygen consumption. AB1133(pPAV01) and AB1133(pPAV01, pALS13) strains were grown to exponential phase (OD_{600} = 0.4–0.5) in M9-glucose supplemented with 0.2% tryptone. Samples were diluted in the same medium to an OD_{600} of 0.2, followed by incubation at 37°C during 20 min without MccJ25 (control) and with either 10 or 20 μM concentrations of the antibiotic. The average rate of cell respiration over the subsequent 5 min was polarographically measured with a Clark-type electrode oxygraph and normalized to the OD_{600}.

RESULTS

MccJ25 susceptibility in *spoT* mutant strains with increments in ppGpp basal levels. During studies on the response of *E. coli* strains to MccJ25 we noted that cells taken from the early stationary phase were more resistant to the antibiotic than those in the log-phase of growth. After entry into stationary phase, the level of ppGpp is known to increase (9). Therefore, we tested whether susceptibility to MccJ25 is affected by changes in the level of ppGpp by using a series of mutant strains which vary in their ability to produce the alarmone: CF4941 (Δ*relA*), CF4977 (Δ*relA spoT202*), and CF4943 (Δ*relA spoT203*). The last two strains were derived by mutating the *spoT* gene (which encodes the major cellular ppGppase) so as to obtain progressive increments in ppGpp basal levels (36). Alleles *spoT202* and *spoT203* confer incremental ppGpp basal level elevations of about six- and eightfold, respectively, compared to the parent strain CF4941 (36). The susceptibility of

TABLE 2. Sensitivity to MccJ25

Strain	MccJ25 MIC (μg/ml)[a]
MC4100	15.6
RO98	0.2
AB1133	1.9
AB1133(pALS13)	7.8
MC4100(pALS13)	31.2
AB1133(pCNB0209R)	31.2

[a] The experiment was repeated five times with similar results.

the isogenic mutant strains was compared to that of the control CF4941 in a spot test, as described in Materials and Methods. Strain CF4941 gave slightly turbid halos until a microcin dilution of 1:64, whereas for strains CF4943 and CF4977 the microcin titer was the same (1:64), but the spots were much more turbid. Interestingly, turbidity was correlated with the increments in ppGpp basal levels. In fact, for strain CF4977 the halos were hardly visible. When the mutant *E. coli* CF1693 (Δ*relA* Δ*spoT*), which is unable to synthesize ppGpp, was tested, it gave clear halos of inhibition. Thus, in a null ppGpp background, intrinsic resistance to microcin was completely lost.

We observed that strain RO98 (Δ*relA* Δ*spoT*), a MC4100 derivative that is also devoid of ppGpp, shows an increased sensitivity to MccJ25 (78-fold) compared to the parent strain (Table 2). These results indicate a direct correlation between intrinsic resistance of several *E. coli* strains to MccJ25 and the ppGpp production ability of the bacteria.

Overproduction of ppGpp from a plasmid bearing a truncated *relA* gene induces resistance to microcin J25. An alternative method for increasing ppGpp in unstarved cells involves abrupt induction to levels approximating those achieved during amino acid or carbon source starvation. This method uses a plasmid, pALS13, bearing the *relA* gene fused to a *tac* promoter (41). With this plasmid, the overproduction of ppGpp can be induced in a controlled fashion with IPTG (isopropyl-β-D-thiogalactopyranoside); i.e., the degree of ppGpp overproduction is directly related to the concentration of IPTG used for the induction of *relA*. After IPTG induction of the *tac* promoter cells containing pALS13 accumulate ppGpp in the range normally observed during the stringent response (36, 37, 41). The plasmid was transformed into *E. coli* strain AB1133 (which was chosen because it is naturally highly susceptible to MccJ25) and strain MC4100. These strains proved to be very sensitive to ppGpp levels. In fact, induction of RelA expression from plasmid pALS13, even with as low a concentration of IPTG as 0.05 mM, was found to have severe effects on growth. Since this could complicate interpretation of the results, in all of the ppGpp accumulation experiments we decided to omit the inducer from the test medium. As shown here, the basal level of *relA* expression caused by the leakiness of the P_{tac} promoter from the multicopy plasmid was sufficient to confer microcin resistance. As seen in Table 2, the MICs of MccJ25 for AB1133(pALS13) and MC4100(pALS13) were four- and twofold higher, respectively, than those observed for the plasmidless controls.

We also compared growth and viability of strains AB1133 (pALS13) and AB1133 in the presence and in the absence of microcin. Both strains were grown in LB to an OD_{600} of 0.1 to 0.2, when the cultures were divided and half was treated with 2 μM microcin. Samples were taken at intervals, a portion was used to measure OD_{600}, and the rest of sample diluted into sterile M9 medium and plated on LB for viable counts. As can be seen in Fig. 1, after 2 h of MccJ25 addition the viable-cell counts for strain AB1133 had dropped from 10^8 to about 10^6 CFU/ml. After 4 h of exposure to microcin, viability had decreased 1 order of magnitude more. In contrast, MccJ25 did not inhibit cell growth or viability of AB1133(pALS13), indicating that elevated intracellular amounts of ppGpp were accompanied by a protective effect against MccJ25. The fact that uninduced levels of ppGpp sufficed to cause microcin resis-

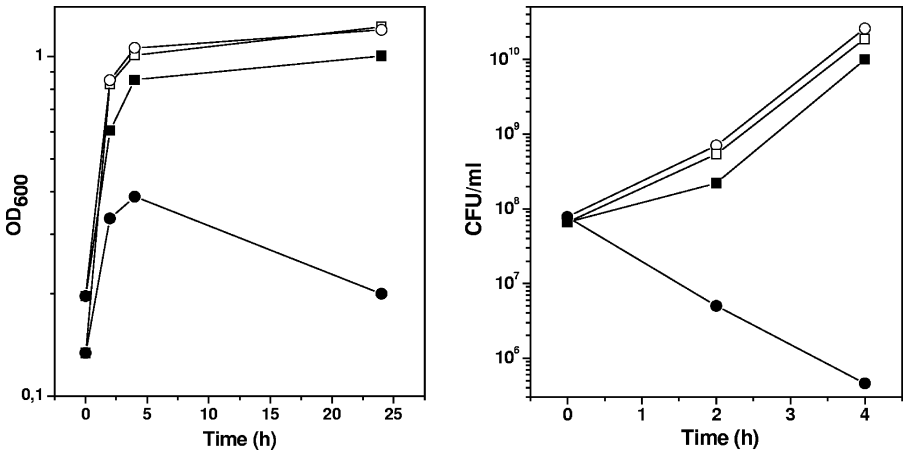

FIG. 1. Effect of ppGpp on inhibition of culture growth and cell viability caused by MccJ25. Strains AB1133 (circles) and AB1133(pALS13) (squares) were grown at 37°C in LB and LB plus ampicillin, respectively. At time zero, the cultures were split such that one-half received 2 μM MccJ25 (filled symbols), whereas the other half remained untreated (open symbols). Aliquots of each culture were withdrawn at the indicated time points for measurement of cell growth (left panel) and viability (right panel). For viable counts, the cultures were serially diluted into sterile M9 medium and plated on LB agar. The number of colonies obtained after 24 h of incubation at 37°C was used to establish the survival curves. The experiment was done three times with similar results. The values shown are from one representative experiment.

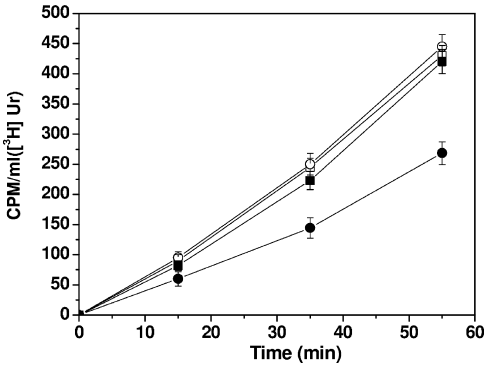

FIG. 2. Effect of ppGpp on the inhibition of RNA synthesis by MccJ25. Strains AB1133 (circles) and AB1133(pALS13) (squares) were grown at 37°C in M9-glucose and M9-glucose plus ampicillin, respectively. At time zero, the cultures were divided into equal portions, one of which received MccJ25 to a final concentration of 0.8 μM (filled symbols), while the other was left untreated (open symbols). Aliquots of each were withdrawn into ice-cold trichloroacetic acid at the indicated time points, and the incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material was measured as described in Materials and Methods. Error bars represent standard deviations from five experiments.

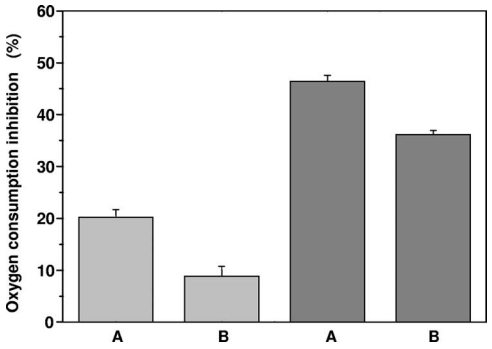

FIG. 3. Effect of ppGpp on the inhibition of cell respiration by MccJ25. Strains AB1133(pPAV01) (A) and AB1133(pPAV01, pALS13) (B) were grown in minimal medium M9-glucose supplemented with the appropriate antibiotics for plasmid maintenance and tryptone (0.2%). The latter was added to allow growth of the poliauxotrophic strain AB1133. Respiratory rate was determined in the presence of 10 μM (light gray bars) or 20 μM (dark gray bars) MccJ25 as described in Materials and Methods, and the results are expressed as the percent respiratory inhibition relative to the controls in the absence of MccJ25. Error bars represent standard deviations from five experiments.

tance indicates that there is a low threshold concentration of ppGpp required to initiate, or increase, intrinsic resistance to MccJ25.

High ppGpp levels do not induce a microcin sequestering or modifying activity. The decreased susceptibility to MccJ25 in the presence of elevated levels of ppGpp may result from this nucleotide inducing a MccJ25-modifying activity or the synthesis of a molecule capable of sequestering the antimicrobial agent in the cytoplasm or periplasm, thus preventing it from reaching its sites of action. If so, we predicted that these molecules should physically interact in vitro. To test this, we examined interactions of crude cell extracts with MccJ25. Stationary-phase LB cultures of AB1133 and AB1133(pALS13) were centrifuged, and the cell pellets were lysed with a French press. Portions (100 μl) of cell lysates were mixed with MccJ25 (final concentration, 235 μM) and, after a 4-h incubation at 37°C, the antibiotic activity titer was determined in a spot-on-lawn test. No difference was found between the control mixture (AB1133) and that containing the AB1133(pALS13) lysate. Thus, no evidence of microcin sequestration or loss of activity was obtained.

High ppGpp levels prevent MccJ25 from inhibiting its targets of action. It is well known that MccJ25 targets RNA polymerase and inhibits transcription (13). More recently, we have found that in *E. coli* cells overproducing the microcin receptor, FhuA, the antibiotic also inhibits cell respiration (5). The decreased susceptibility to MccJ25 of cells with high ppGpp levels suggests that one or both of these targets are somehow protected from the antibiotic action. To verify this, we performed in vivo transcription experiments with strains AB1133 and AB1133(pALS13) in the presence or absence of microcin. Exponentially growing cells of AB1133 and AB1133 (pALS13) were treated with 0.8 μM microcin, and RNA synthesis was measured as described in Materials and Methods. As expected, RNA accumulation in strain AB1133 was reduced by ca. 40% after a 60-min treatment with microcin (Fig. 2). Under these conditions, AB1133(pALS13) continued to accumulate RNA to a level comparable to that of AB1133(pALS13) in the absence of microcin (Fig. 2). Thus, high ppGpp levels prevented the inhibition of RNA synthesis by MccJ25.

We also measured oxygen consumption of strain AB1133 transformed with plasmid pPAV01 (which overexpresses the *E. coli fhuA* gene) and compared it to that of the same strain cotransformed with pPAV01 and pALS13. In the presence of 10 μM MccJ25 strain AB1133(pPAV01) showed the expected inhibition of respiration, whereas in the doubly transformed strain the accumulation of ppGpp restored oxygen consumption to about half the normal levels shown by strain AB1133(pPAV01) (Fig. 3). Note that in contrast to what is observed for RNA polymerase activity, the protection effect of ppGpp on cell respiration is not complete. Moreover, when this experiment was performed with 20 μM MccJ25 a lower effect (20%) was seen (Fig. 3). A likely explanation is that the increase in the intracellular accumulation of microcin resulting from overexpression of its receptor partially overrides the ppGpp effect. This hypothesis was substantiated by the fact that we obtained a partial protective action of ppGpp on the growth of AB1133(pPAV01, pALS13) compared to the complete protection seen for AB1133(pALS13) (data not shown).

Protective action of ppGpp results of an increased MccJ25 export. The question arises as to how the intracellular accumulation of ppGpp leads to an increased expression of MccJ25 intrinsic resistance in *E. coli* cells. The fact that elevated levels of the alarmone protects not only RNA polymerase but also the other target of MccJ25, the respiratory chain, suggests that this may not be a target-specific mechanism but is more likely to be a general mechanism, e.g., increased expression of an efflux transport protein. As stated in the introduction, immunity against MccJ25 in producer cells relies on a plasmid-borne dedicated ABC exporter to minimize the intracellular concentrations of the antibiotic. Recently, we demonstrated that YojI, an *E. coli* chromosomal protein with ATP-binding-cassette-type exporter homology (27), is able to export MccJ25 out of

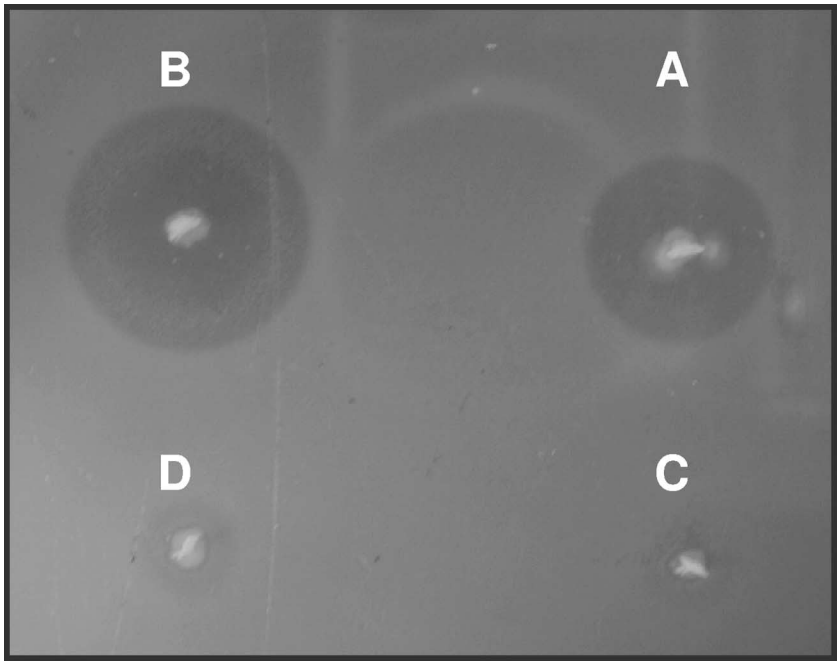

FIG. 4. Effect of ppGpp on MccJ25 export by YojI. Microcin-resistant strain SBG231 containing pFP348 (A) or both pFP348 and pALS13 (B), and the corresponding deletion strains lacking *yojI* (panels C and D, respectively) were assayed for production of extracellular MccJ25. Plasmid pFP348 directs MccJ25 synthesis but lacks the functional MccJ25 export-immunity gene *mcjD*. Isolated colonies were stabbed into M9-glucose plates and pregrown for 12 h before they were killed with chloroform, overlaid with a lawn of sensitive cells and incubated overnight at 37°C. See the text for details.

the cells (15). The physiological role of YojI is not yet understood. One attractive explanation for the effect of ppGpp on microcin resistance is that it could be mediated or characterized by the upregulation of the YojI efflux mechanism, which would keep the intracellular concentration of MccJ25 below a toxic level. To test this possibility, strain SBG231, containing a MccJ25-resistant RNA polymerase (13), was transformed with plasmid pFP348, which carries the *mcjABC* MccJ25 production genes but not the *mcjD* immunity gene. The transformants grew normally, indicating that the mutation overcame the otherwise lethal effect of accumulated internal MccJ25, and they were able to give a growth inhibition halo on a MccJ25-sensitive indicator strain (Fig. 4, A). In the absence of the natural MccJ25 exporter, McjD, this export activity can be ascribed to YojI. Increasing the ppGpp levels by introduction of plasmid pALS13 led to a significantly enhanced MccJ25 secretion (Fig. 4, B). In the absence of YojI, a faint, tiny zone of inhibition was visible, either under normal or plasmid-increased levels of ppGpp pool (Fig. 4, C and D, respectively), possibly due to a nonspecific leakage of microcin out of the cells. Taken together, these results may be explained by assuming that ppGpp stimulates YojI expression or activity.

ppGpp positively regulates the *yojI* gene. The finding that ppGpp increases microcin export by YojI suggested that ppGpp could positively regulates *yojI* expression. To test this, a transcriptional *yojI-lacZ* fusion was constructed as described in Materials and Methods, generating strain SBS100. The fusion activity was measured under conditions of either deficient or elevated ppGpp levels, both in exponential and in stationary phase. High intracellular concentrations of ppGpp were achieved by transforming SBS100 with plasmid pALS13, whereas for a ppGpp-deficient background, a Δ*spoT207*::*cat* allele was transduced into SBS100 (which already contains a *relA1* mutation [23]). The results are shown in Fig. 5 and may be summarized as follows. First, under normal physiological conditions, the expression of *yojI* is higher in stationary phase (compare the "B" bars in both phases in the figure). Second, in a ppGpp-

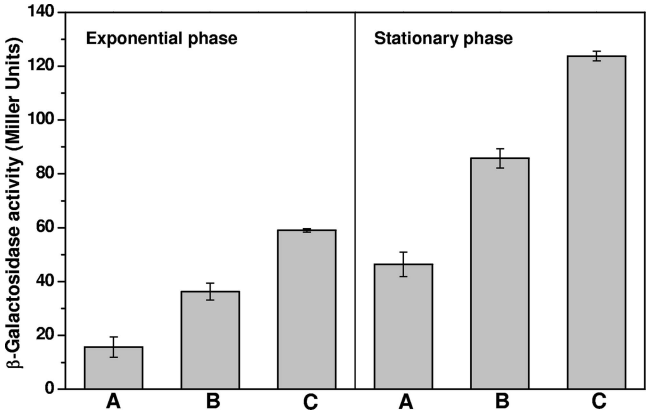

FIG. 5. Effect of ppGpp on *yojI*::*lacZ* expression. Strains SBS100 (*yojI*::*lacZ*) (B), SBS100 *spoT* (ppGpp deficient) (A), and SBS100 (pALS13) (high ppGpp) (C) were grown in LB, and *yojI*::*lacZ* expression was measured by determining the β-galactosidase activity in the exponential and stationary phases of growth. β-Galactosidase activity values, shown as Miller units, are averages from at least six replicates.

deficient background, *yojI* transcriptional activity dropped to about half of the value seen for the control SBS100, in the log as well in stationary phase of growth. Last, introduction of pALS13 into strain SBS100 increased *yojI* expression in the log and stationary phases by 63 and 44%, respectively. From these results, we conclude that ppGpp upregulates *yojI* expression.

A plasmid bearing a full-length *relA* gene is more effective at inducing resistance to MccJ25 than pALS13. When this study was near to completion, a plasmid, pCNB0209R, carrying a complete *relA* gene was made available to us by Victor de Lorenzo. This plasmid carries a His-tagged variant of the *relA* gene of *E. coli*, whose expression is tightly controlled by a *lacI*/P_{lac} system (8). We wanted to compare the microcin resistance of pALS13- and pCNB0209R-bearing cells. *E. coli* AB1133 was transformed with pCNB0209R and the MIC for MccJ25 was measured (as with pALS13, the experiment was carried out without the addition of IPTG). The data in Table 2 show that cells containing the pCNB0209R plasmid were even more resistant to MccJ25 than those transformed with pALS13. This provides confirmatory evidence of the results obtained with the latter plasmid.

DISCUSSION

In the present study, we demonstrate that there exists a direct correlation between intrinsic resistance of *E. coli* to microcin J25 and the intracellular pool of ppGpp. This was accomplished by examining the effect of artificially manipulating the ppGpp levels on MccJ25 resistance.

Tuomanen (43) reported that the *relA* gene product appeared to be involved with the induction of phenotypic tolerance to β-lactam antibiotics in *E. coli*. Gilbert et al. (18) reviewed the influence of growth rate and antimicrobial resistance in biofilms and reported that the stringent response may be a major factor in determining increased resistance of bacteria at slow growth rates. It has also been reported that the killing effect of penicillin is reduced in amino acid-starved *E. coli* if the cells are also producing ppGpp (30). Resistance to the related antibiotic, amdinocillin, can also be observed in *E. coli* with low growth rates or if ppGpp levels are artificially raised (20, 45). These results indicate that low growth rates and ppGpp accumulation in *E. coli* can influence the cell's resistance to antimicrobial agents. Our work extends those of these authors to include MccJ25, a completely different kind of antibiotic. Our results show that the role of ppGpp in microcin intrinsic resistance involves a stimulation of the expression of the YojI efflux pump, which, by pumping out microcin molecules, would reduce the intracellular levels of the antibiotic. It remains to be seen whether the ppGpp-dependent increase in *yojI* transcriptional activity in vivo reflects a direct or indirect effect of the alarmone.

Although it is clear that ppGpp stimulates the expression of *yojI*, such a stimulation was, however, fairly moderate and perhaps insufficient to fully account for the observed increase in microcin resistance. Therefore, there is probably not one single mechanism to explain all of the ppGpp's effect. In this regard, we can suggest other mechanisms unrelated to efflux that could potentially contribute to microcin resistance. First, multiple lines of evidence support the conclusion that MccJ25 inhibits transcription by binding within and sealing off the RNAP secondary channel (like a cork in a bottle), thus blocking nucleotide entry into the active site (1, 25). In addition, recent work from several groups has shown that the secondary channel can be exploited by regulatory factors to gain access to the RNAP catalytic center (26). When bound within the secondary channel, these factors affect transcription, either by modifying the catalytic properties of RNAP or by potentiating the action of small molecule effectors. An example of this last effect is that exerted by the transcription factor DksA, which may directly stabilize the binding of ppGpp to RNAP, enhancing its effect on rRNA transcription in vitro (28, 29). Thus, one can speculate that high ppGpp levels might favor DksA binding and that this, in turn, would block access of MccJ25 to its binding pocket. This would desensitize the RNAP to the effect of the antibiotic.

Second, although it is clear that RNAP is the target for ppGpp interaction, unequivocal proof where exactly the effector molecule binds to *E. coli* RNAP is still pending. In this connection, a recent study by Vrentas et al. (46) casts doubt on the biological relevance of the ppGpp binding site as defined by the *Thermus thermophilus* RNAP-ppGpp cocrystal structures (2). This being the case, it is even possible that ppGpp and MccJ25 may compete against each other in binding to RNAP. Alternatively, ppGpp could be an allosteric effector that interacts at some site within the secondary channel (or elsewhere) and triggers a conformational change, which alters the complex interacting surface that is known to make contacts with MccJ25 (25).

Although our concern here has been with resistance to exogenous microcin, the results described here might have evolutionary implications regarding resistance to endogenous antibiotic in MccJ25-producing cells. We can imagine that, since the MccJ25 system evolved, YojI preceded McjD in the export of MccJ25 (or a structural forerunner). This idea does not imply that MccJ25 was the natural substrate for YojI. The increased levels of YojI in stationary phase must have been advantageous for the producing bacteria, since it is in this phase when production of MccJ5 is maximal. It is conceivable that the early microcin synthetic apparatus was inefficient and that, concurrently with its evolution to become a more efficient machine, YojI was displaced by a more active and specific exporter, McjD.

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