Properties of a Revertant of *Escherichia coli* Viable in the Presence of Spermidine Accumulation: Increase in L-Glycerol 3-Phosphate

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Escherichia coli CAG2242 cells are deficient in the *speG* gene encoding spermidine acetyltransferase. When these cells were cultured in the presence of 0.5 to 4 mM spermidine, their viability was greatly decreased through the inhibition of protein synthesis by overaccumulation of spermidine. When the cells were cultured with a high concentration of spermidine (4 mM), a revertant strain was obtained. We found that a 55-kDa protein, glycerol kinase, was overexpressed in the revertant and that synthesis of a ribosome modulation factor and the RNA polymerase σ^{38} subunit, factors important for cell viability, was increased in the revertant. Levels of L-glycerol 3-phosphate also increased in the revertant. Transformation of *glpFK*, which encodes a glycerol diffusion facilitator (*glpF*) and glycerol kinase (*glpK*), to *E. coli* CAG2242 partially prevented the cell death caused by accumulation of spermidine. It was also found that L-glycerol 3-phosphate inhibited spermidine binding to ribosomes and attenuated the inhibition of protein synthesis caused by high concentrations of spermidine. These results indicate that L-glycerol 3-phosphate reduces the binding of excess amounts of spermidine to ribosomes so that protein synthesis is recovered.

Polyamines (putrescine, spermidine, and spermine) are necessary for normal cell growth, and their proliferative effects are probably due to stimulation of nucleic acid and protein synthesis (3, 24). A decrease in polyamine content causes a decrease in the rate of cell proliferation and protein synthesis (12, 34). Furthermore, overaccumulation of polyamines can inhibit protein synthesis and cell growth (7, 23). Thus, the optimal concentrations of polyamines are necessary for protein synthesis and cell growth. *Escherichia coli* mutants with disruptions of enzymes in the polyamine synthetic or degradative pathways are useful systems with which to probe the physiological roles of polyamines. One such mutant lacks the *speG* gene encoding spermidine acetyltransferase, which catalyzes the first step of polyamine degradation in *E. coli* (5). In the *speG* mutant, we found that addition of spermidine to the medium reduces cell viability in the late stationary phase due to intracellular accumulation of spermidine (6). The accumulation of spermidine caused a marked decrease in protein synthesis but not in DNA and RNA synthesis in the stationary phase (6).

The synthesis of several kinds of proteins was particularly inhibited by spermidine in the late stationary phase in the *speG* mutant. These proteins include a ribosome modulation factor (RMF) (6) and the RNA polymerase stationary-phase-specific sigma subunit σ^S or σ^{38} (1). RMF is synthesized in the stationary phase and is uniquely associated with 100S dimer ribosomes (27, 28). A mutant lacking RMF showed decreased cell viability in the stationary phase (32). The σ^{38} subunit is in-

volved in the transcription of a group of genes for stationary-phase survival and stress response to heat shock or osmotic shock (8, 15, 33).

In this study, we isolated a revertant strain of the *speG* mutant which can survive during growth in a high concentration of spermidine. Although polyamines were accumulated in the revertant, the synthesis of RMF and the σ^{38} subunit was increased, apparently due to inhibition of spermidine binding to ribosomes. Inhibition of polyamine binding to ribosomes was due to an increase in L-glycerol 3-phosphate, which interacts with spermidine.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *E. coli* CAG2242 (*speG supE44 hsdR thi thr leu lacY1 tonA21*), a spermidine acetyltransferase-deficient mutant (2), was kindly supplied by E. W. Gerner (University of Arizona Health Sciences Center). The cells were grown in a modified Luria-Bertani (LB) medium (8 g of tryptone per liter, 4 g of yeast extract per liter, and 5 g of NaCl per liter supplemented with 1 mM sodium phosphate, pH 7.4). Where indicated, 2 or 4 mM spermidine was added at the onset of cell growth. Cell growth was monitored by measuring A_{540}. Cell viability was determined by counting colonies in aliquots of the culture grown on an LB medium-containing 1.5% agar plate at 37°C. Thus, the definition of viable cells is cells that are able to grow on an agar plate. When pUC118- or -119-derived plasmids were transformed, 100 μg of ampicillin per ml was added to the medium.

Measurement of polyamines, L-glycerol 3-phosphate, and protein contents. Polyamine levels in *E. coli* were determined by high-pressure liquid chromatography as described previously (10) after homogenization and extraction of the polyamines with 5% trichloroacetic acid and centrifugation at 27,000 × *g* for 15 min at 4°C. Levels of L-glycerol 3-phosphate were measured by the method of Hohorst (9). The reaction mixture (1 ml), consisting of 0.18 M hydrazine, 0.45 M glycine buffer (pH 9.5), 2.5 mM β-NAD, 60 μg of α-glycerophosphate dehydrogenase (Sigma), and 0.2 ml of extract obtained as described above after neutralization with ether, was incubated at 24°C for 30 min, and A_{334} was measured. Levels of L-glycerol 3-phosphate were calculated from the calibration curve of

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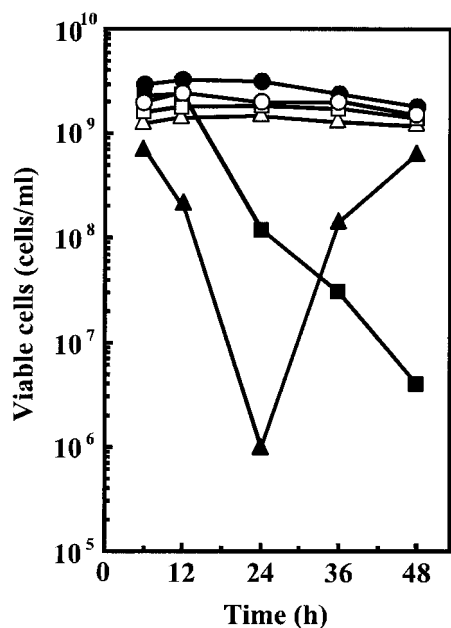
FIG. 1. Effect of spermidine on cell viability of *E. coli* CAG2242 and its revertants. Viable cells were counted at the designated times as described in Materials and Methods. Symbols for *E. coli* CAG2242: ●, no spermidine; ■, 2 mM spermidine; ▲, 4 mM spermidine. Revertant cells (▲) were collected after 48 h of culture in the presence of 4 mM spermidine and cultured again under the following conditions: ○, no spermidine; □, 2 mM spermidine; △, 4 mM spermidine. Each point is the average of duplicate determinations.

FIG. 3. Effect of spermidine on cellular levels of putrescine (A) and spermidine (B). Polyamine contents were measured at the designated times as described in Materials and Methods. *E. coli* CAG2242 and SR-199 cells were cultured in the absence (□) and presence (■) of 4 mM spermidine. Each value is the average of duplicate determinations.

L-glycerol 3-phosphate. Protein was determined by the method of Lowry et al. (19).

Western blot analysis. Antibody for RMF was made by injecting 1 mg of RMF with Freund's complete adjuvant into a rabbit. Antibody for the RNA polymerase σ^{38} subunit was prepared as described previously (16). Western blotting was performed by the method of Nielsen et al. (21).

Identification of induced protein in the revertant by protein sequencing. Induced protein in the revertant was identified by electrophoresis on a sodium dodecyl sulfate (SDS)–10.5% polyacrylamide gel (18). After blotting, the induced protein was analyzed by automated Edman degradation on a protein sequencer (Applied Biosystems model 470A) equipped with a phenylthiohydantoin analyzer (model 120A).

Construction of pUC*glpFK*, pUC*glpFKX*, pUC*glpF*, pUC*glpK*, and pUC*glpX*. PCR was performed by using total chromosomal DNA as a template and 5′-G GCCTGCAGACGTTGCTGCCAGCCGTTCTG-3′ (P1) and 5′-TGGGTCGA CGTGTAGCACAGGGGAAGGGAG-3′ as primers to obtain the *glpFK* gene (22, 30). pUC*glpFK* was constructed by inserting the 3.2-kb *Pst*I-*Sal*I fragment of the PCR product into the same restriction sites of pUC118 (TaKaRa). PCR was also performed by using total chromosomal DNA as a template and P1 and 5′-CCTGTCGACATTGACTTGCCTTATCTTCGT-3′ (P2) as primers to obtain the *glpFKX* gene. pUC*glpFKX* was constructed by inserting the 4.3-kb *Pst*I-
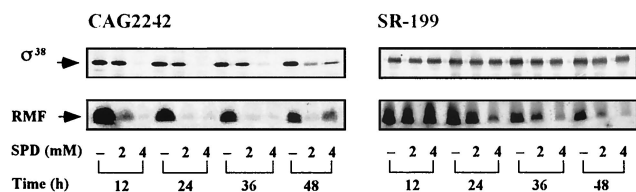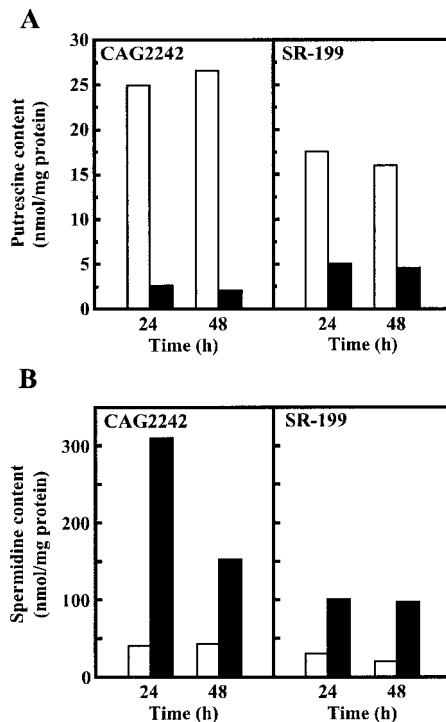

FIG. 2. Effect of spermidine (SPD) on the synthesis of the σ^{38} subunit and RMF in *E. coli* CAG2242 and its revertant SR-199. Western blotting was performed by using 5 µg of protein for σ^{38} and 25 µg of protein for RMF.

*Sal*I fragment of the PCR product into the same restriction sites of pUC118. pUC*glpF-1* and pUC*glpF-2* (isopropyl-β-D-thiogalactopyranoside [IPTG] inducible) were constructed by deleting the 1.9-kb *Sma*I-*Nru*I fragment of pUC*glpFK* and by inserting the 1.4-kb *Sph*I-*Nru*I fragment of pUC*glpFK* into the *Sph*I-*Sma*I sites of pUC119 (TaKaRa), respectively. pUC*glpK* (IPTG inducible) was constructed by inserting the 1.6-kb *Hin*dIII-*Sal*I fragment of pUC*glpFK* into the same restriction sites of pUC119. PCR was performed by using total chromosomal DNA as a template and 5′-GTGCTGCAGTTCGCGCCATTCCTTACT GCT-3′ and P2 as primers to obtain the *glpX* gene. pUC*glpX* (IPTG inducible) was constructed by inserting the 1.1-kb *Pst*I-*Sal*I fragment of the PCR product into the same restriction sites of pUC119.

Assays for polyphenylalanine synthesis and spermidine binding to ribosomes. Salt-washed ribosomes, the 0.25 M NH_4Cl fraction of DEAE-cellulose column chromatography of S100, containing aminoacyl-tRNA synthetases and elongation factors, and tRNA mixtures were prepared from *E. coli* Q13 essentially as described previously (11, 29). Polyphenylalanine synthesis was measured by using 15 µg of salt-washed ribosomes in the presence of 12 mM Mg^{2+} and 100 mM NH_4^+ as described previously (27). The reaction mixture (0.1 ml) for spermidine binding to ribosomes contained 50 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, 30 mM NH_4Cl, 150 µg of salt-washed ribosomes, and 0.25 mM [^{14}C]spermidine (1.85 kBq). After incubation for 10 min at 30°C, the reaction mixture was chilled and passed through a cellulose nitrate membrane filter (Advantec). The filter was washed with a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, and 30 mM NH_4Cl, and the radioactivity on the filter was counted with a liquid scintillation counter.

RESULTS

Isolation of a revertant viable in the presence of spermidine accumulation. We previously reported that *E. coli* CAG2242, a *speG* mutant, showed accumulation of spermidine and a subsequent decrease in cell viability in the late stationary phase of

A

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        CAG2242   SR-199
kDa

94 —

68 —

                          ◄

45 —
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B

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  1 MTEKKYIVAL DQGTTSSRAV VMDHDANIIS
 31 VSQREFEQIY PKPGWVEHDP MEIWATQSST
 61 LVEVLAKADI SSDQIAAIGI TNQRETTIVW
 91 EKETGKPIYN AIVWQCRRTA EICEHLKRDG
121 LEDYIRSNTG LVIDPYFSGT KVKWILDHVE
151 GSRERARRGE LLFGTVDTWL IWKMTQGRVH
181 VTDYTNASRT MLFNIHTLDW DDKMLEVLDI
211 PREMLPEVRR SSEVYGQTNI GGKGGTRIPI
241 SGIAGDQQAA LFGQLCVKEG MAKNTYGTGC
271 FMLMNTGEKA VKSENGLLTT IACGPTGEVN
301 YALEGAVFMA GASIQWLRDE MKLINDAYDS
331 EYFATKVQNT NGVYVVPAFT GLGAPYWDPY
361 ARGAIFGLTR GVNANHIIRA TLESIAYQTR
391 DVLEAMQADS GIRLHALRVD GGAVANNFLM
421 QFQSDILGTR VERPEVREVT ALGAAYLAGL
451 AVGFWQNLDE LQEKAVIERE FRPGIETTER
481 NYRYAGWKKA VKRAMAWEEH DE
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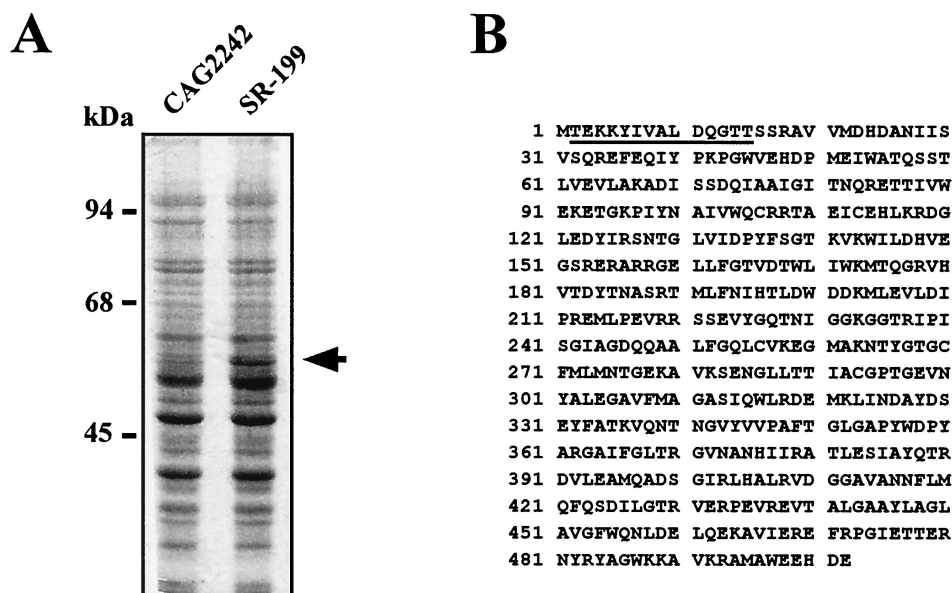
FIG. 4. SDS-polyacrylamide gel electrophoresis of proteins from *E. coli* CAG2242 and SR-199 (A) and amino acid sequence of the NH₂ terminus of the induced protein in *E. coli* SR-199 (B). (A) *E. coli* CAG2242 and SR-199 were cultured in the absence and presence of 4 mM spermidine, respectively, for 24 h. SDS-polyacrylamide gel electrophoresis was performed with 20 μg of protein. The arrow indicates the induced protein. (B) The amino acid sequence determined by Edman degradation is underlined. Accordingly, the induced protein was identified as a glycerol kinase, whose amino acid sequence is shown in panel B.

cell growth when cells were cultured in the presence of spermidine (0.5 to 2 mM) (1, 6). When cells were cultured in the presence of a high concentration of spermidine (4 mM), cell viability was drastically decreased until 24 h after the onset of cell growth (Fig. 1). However, viable revertants were obtained at 36 to 48 h after the onset of cell culture (Fig. 1). When these revertants were recultured in the presence of 4 mM spermidine, the revertants were resistant to cell death caused by spermidine accumulation (Fig. 1). We isolated 10 revertant colonies and studied them further. It was first determined whether RMF and the σ³⁸ subunit, factors important for cell viability, can be synthesized by the revertants. All of the revertants could synthesize both RMF and the σ³⁸ subunit until 48 h after the onset of cell culture in the presence of 4 mM spermidine, although synthesis of RMF gradually decreased as cell culture progressed. In Fig. 2, results of experiments done with one of the revertants (*E. coli* SR-199) are shown.

The intracellular polyamine contents of *E. coli* CAG2242 and the revertant SR-199 were measured after cells were cultured in the presence and absence of 4 mM spermidine. At 24 h after the onset of cell culture in the presence of 4 mM spermidine, the spermidine content of *E. coli* SR-199 was about one-third of that of *E. coli* CAG2242 (Fig. 3B) but a significant amount of spermidine still accumulated in *E. coli* SR-199. At 48 h, the difference in spermidine level between the two strains became about two-thirds due to a decrease in the spermidine level of *E. coli* CAG2242. (Fig. 3B). When spermidine accumulated in cells, the putrescine content decreased greatly, probably due to inhibition of the synthesis of ornithine decarboxylase (Fig. 3A and B). Similar results were obtained with the other nine revertants.

Significant amounts of spermidine accumulate in *E. coli* SR-199, but this accumulation is not cytotoxic. This suggests that a

substance which disturbs the interaction between ribosomes and polyamines may be induced in the revertant, and subsequently the inhibition of protein synthesis due to overaccumulation of spermidine may be prevented.

Induction of the *glpFK* operon in the revertant. We examined the proteins induced in the revertant *E. coli* SR-199 by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4A, a protein of about 55 kDa was strongly induced in the revertant cultured with 4 mM spermidine. A protein of the same molecular mass was also induced in the other nine revertants. The amino acid sequence of the induced protein was determined by Edman degradation, and the NH₂-terminal 14 amino acid residues were identified (Fig. 4B). Based on this sequence, the protein was identified as glycerol kinase. Induction of glycerol kinase was also observed in *E. coli* SR-199 cultured without spermidine (data not shown).

We next cloned the *glpFK* operon encoding a glycerol diffusion facilitator and glycerol kinase (22, 31) by PCR and determined whether expression of *glpFK* could prevent the cytotoxic effect of spermidine accumulation. As shown in Fig. 5, the time course of the decrease in cell viability was greatly changed in *E. coli* CAG2242 transformed with the vector pUC118 or -119 (Fig. 1). Maximal cell death was observed at 60 h during a 60-h culture; that is, revertants viable during spermidine accumulation were not obtained. However, the mechanism of this effect is unknown. When *glpFK* was transformed to *E. coli* CAG2242, cell viability recovered greatly. Either *glpF*, encoding a glycerol diffusion facilitator, or *glpK*, encoding glycerol kinase, partially restored cell viability, so the two genes functioned together. In relation to this finding, it has been reported that glycerol kinase is activated by interaction with the glycerol facilitator (26). The level of glycerol kinase in *E. coli* CAG2242/
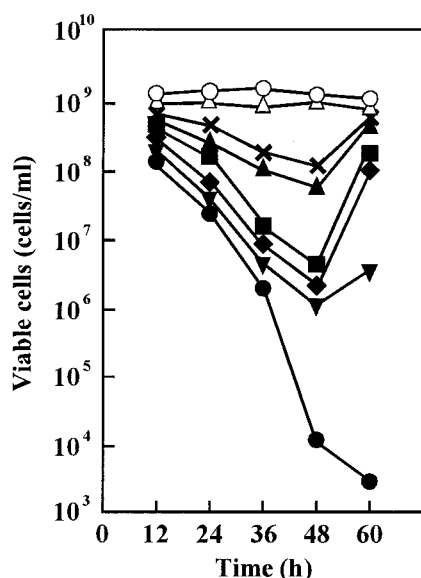
FIG. 5. Effect of *glpFKX* on *E. coli* CAG2242 cell viability. Viable cells were counted at the designated times as described in Materials and Methods. Cells transformed with the indicated plasmids were cultured in the absence and presence of 4 mM spermidine. Symbols: ○, pUC119 and no spermidine; △, pUC*glpFK* and no spermidine; ●, pUC119 and 4 mM spermidine; ◆, pUC*glpF* and 4 mM spermidine; ■, pUC*glpK* and 4 mM spermidine; ▼, pUC*glpX* and 4 mM spermidine; ▲, pUC*glpFK* and 4 mM spermidine; ×, pUC*glpFKX* and 4 mM spermidine. When the gene was oriented under the control of the *lac* promoter, 1 mM IPTG was added at 6 h after the onset of cell culture. In the case of *glpF*, essentially the same results were obtained with the plasmids with either the *glpFK* promoter (pUC*glpF-1*) or the *lac* promoter (pUC*glpF-2*). Each point is the average of duplicate determinations.

pUC*glpFK* or *E. coli* CAG2242/pUC*glpK* was slightly higher than that in *E. coli* SR-199 (data not shown).

Recently, the third gene (*X*) of the *glpFK* operon was identified as a gene encoding fructose 1,6-bisphosphatase (4). Since fructose 1,6-bisphosphate is a feedback inhibitor of glycerol kinase (25, 35), the viability of *E. coli* CAG2242 cells cultured with 4 mM spermidine might be more clearly restored if *glpFKX* were transformed to *E. coli* CAG2242 instead of *glpFK*. This possibility was tested, and as shown in Fig. 5, transformation of *glpX* still significantly restored cell viability but the effect was weaker than that obtained by transformation of *glpF* or *glpK*. Furthermore, recovery of cell viability with *glpFKX* was nearly equal to that obtained with *glpFK*, suggesting that involvement of *glpX* in the recovery of cell viability is small.

The levels of L-glycerol 3-phosphate in *E. coli* CAG2242 and the revertant SR-199 were measured. As shown in Table 1, levels of L-glycerol 3-phosphate were much higher in the revertant SR-199 than in *E. coli* CAG2242, confirming that expression of *glpFK* is enhanced in the revertant SR-199. The level of L-glycerol 3-phosphate also increased when *E. coli* CAG2242 was transformed with pUC*glpF*, pUC*glpK*, and pUC*glpFK* (Table 1). It was maximal with *E. coli* CAG2242/pUC*glpFK*, indicating that the level of L-glycerol 3-phosphate correlates with the degree of recovery of cell viability. Addition of 15 mM L-glycerol 3-phosphate also restored cell viability significantly (data not shown).

TABLE 1. Levels of L-glycerol 3-phosphate in various *E. coli* strains

E. coli strain	Culture[a] time (h)	L-Glycerol 3-phosphate concn (nmol/mg of protein)[b]
CAG2242	12	2.74 ± 0.21
	24	2.81 ± 0.14
SR-199	12	6.92 ± 0.21
	24	7.31 ± 0.18
CAG2242/pUC118	24	1.80 ± 0.18
CAG2242/pUC*glpF*	24	4.52 ± 0.11
CAG2242/pUC*glpK*	24	4.61 ± 0.13
CAG2242/pUC*glpFK*	24	5.81 ± 0.14

[a] Cells were cultured in the absence of spermidine.
[b] Each value shown is the mean ± the standard deviation of three determinations.

L-Glycerol 3-phosphate reverses inhibition of protein synthesis caused by overaccumulation of spermidine. We previously reported that polyamines (putrescine and spermidine) have not only a sparing effect on the Mg^{2+} requirement for protein synthesis but also a stimulating effect, which cannot be fulfilled by any amount of Mg^{2+} in the absence of polyamines (14, 29). We also reported that overaccumulation of spermidine inhibits protein synthesis such that cell viability in the stationary phase decreased greatly (6). The inhibition of protein synthesis by overaccumulation of spermidine was mainly due to the inactivation of ribosomes through replacement of Mg^{2+} at magnesium binding sites by polyamines (7, 13). Thus, we examined whether the inhibition of protein synthesis caused by overaccumulation of spermidine was reversed by L-glycerol 3-phosphate by using a cell-free system. As shown in Fig. 6A, polyphenylalanine synthesis at 12 mM Mg^{2+} was inhibited by 45% by 4 mM spermidine. Addition of L-glycerol 3-phosphate gradually recovered the inhibition of polyphenylalanine synthesis. The level of polyphenylalanine synthesis in the presence of 15 mM L-glycerol 3-phosphate and 4 mM spermidine was nearly equal to that in the presence of 2 mM spermidine only. On the other hand, polyphenylalanine synthesis in the absence of spermidine was not influenced by L-glycerol 3-phosphate (Fig. 6A). The effect of L-glycerol 3-phosphate on the binding of [14C]spermidine to ribosomes was also examined. As shown in Fig. 6B, spermidine binding to ribosomes was inhibited by L-glycerol 3-phosphate. The results suggest that L-glycerol 3-phosphate interacts with spermidine so that protein synthesis is recovered by disturbing the binding of spermidine to ribosomes.

DISCUSSION

The results of this study show that L-glycerol 3-phosphate negates the toxicity of spermidine. It presumably does this by disturbing spermidine binding to ribosomes, because it has been already reported that ribosomes are inactivated by an increase in the level of spermidine bound to ribosomes (13, 30). Inactivation of the ribosomes occurred when more than 40% of the Mg^{2+} originally bound to ribosomes was replaced with spermidine (13). One simple explanation is that lower-molecular-mass phosphate compounds, such as L-glycerol 3-phosphate, directly interact with polyamines and thereby modulate their functions. We also reported recently that the
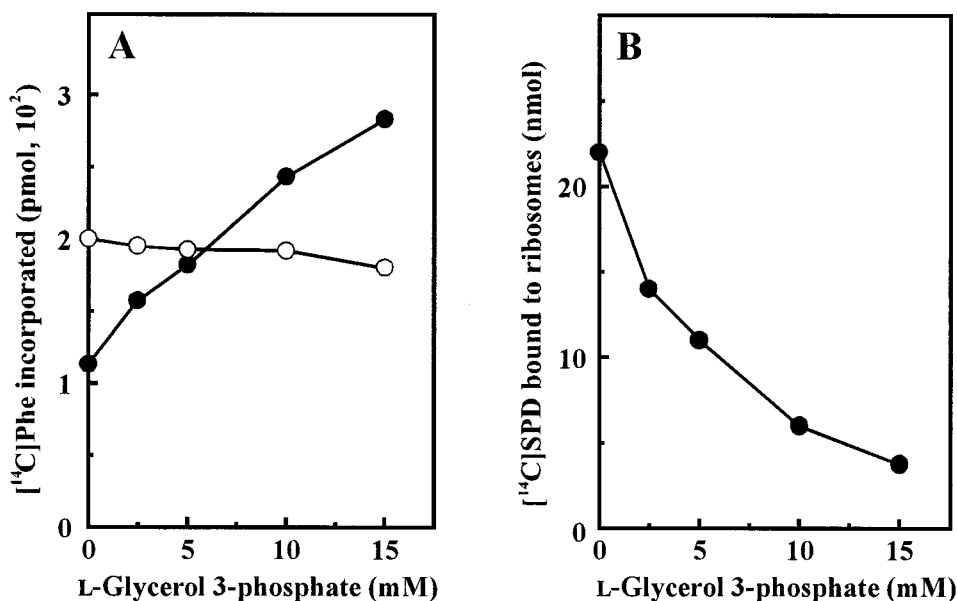
FIG. 6. Effect of L-glycerol 3-phosphate on protein synthesis (A) and spermidine binding to ribosomes (B). Experiments were performed as described in Materials and Methods. (A) Polyphenylalanine (Phe) synthesis at 12 mM Mg^{2+} ($\bigcirc$) and at 12 mM Mg^{2+} and 4 mM spermidine ($\bullet$). (B) Spermidine binding to ribosomes was measured in the presence of 1 mM Mg^{2+} and 0.25 mM spermidine. Each point is the average of duplicate determinations.

function of Mg^{2+}-ATP is modulated by the formation of an Mg^{2+}-ATP-polyamine (spermidine or spermine) complex (20). The ATPase activity of PotA (17) was greatly enhanced by spermine, and the activity of protein kinase A was also stimulated about twofold by spermine (20). However, direct evidence for the interaction between spermidine and L-glycerol 3-phosphate has not been obtained thus far. Thus, another function(s) of L-glycerol 3-phosphate in the recovery of cell viability may also exist.

We have shown that spermidine has not only a sparing effect on the Mg^{2+} requirement for polyphenylalanine synthesis but also a stimulating effect, which cannot be fulfilled by any amount of Mg^{2+} in the absence of spermidine (14). Polyphenylalanine synthesis in the presence of 12 mM Mg^{2+} and 100 mM NH_4^+ was stimulated by 50% by 2 mM spermidine and was inhibited by 45% by 4 mM spermidine (Fig. 6A). Polyphenylalanine synthesis at 4 mM spermidine gradually increased with the increase in L-glycerol 3-phosphate and finally reached the level obtained by 2 mM spermidine. The results support the idea that L-glycerol 3-phosphate disturbs the binding of spermidine to ribosomes, and this is confirmed by the data shown in Fig. 6B. If L-glycerol 3-phosphate similarly functions in the logarithmic phase, it is expected that protein synthesis would be inhibited. However, expression of the *glpK* gene in the logarithmic phase was much weaker than that in the stationary phase (data not shown).

In the revertants, the *glpFK* operon was induced in cells cultured with or without spermidine. This suggests that a mutation may occur in the promoter region of the *glpFK* operon or in a regulatory protein of the operon. We isolated 10 revertant colonies, and the properties of the 10 colonies were indistinguishable, suggesting that they were derived from the same origin. Furthermore, an unidentified membrane protein was

induced only in the cells cultured with spermidine (data not shown). This protein may be involved in the decrease in spermidine at 24 h after the onset of cell culture. The activity of spermidine uptake was almost the same in *E. coli* CAG2242 and the revertant SR-199. Thus, we expect that the protein is involved in the excretion of spermidine from the cells. Since expression of the *glpFK* gene could not restore cell viability completely, the unidentified membrane protein is also important for complete recovery of cell viability. Experiments intended to identify this protein are in progress.

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