Properties of a Revertant of *Escherichia coli* Viable in the Presence of Spermidine Accumulation: Increase in 1-Glycerol 3-Phosphate

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The document contains text related to molecular biology experiments. It describes the construction of plasmids, PCR amplification, and gel electrophoresis to study the effects of spermidine on bacterial viability and polyamine synthesis. The text includes figures showing data on cell viability and spermidine levels over time. It also mentions the isolation of revertants and the use of antibodies for Western blot analysis.

**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 growth. To investigate the role of spermidine in the revertants, we performed PCR amplification using specific primers to detect the presence of the spermidine binding protein RMF. The results showed a significant decrease in spermidine levels in revertants compared to the mutant strain, indicating a reduction in RMF expression.
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 s38 subunit, factors important for cell viability, can be synthesized by the revertants. All of the revertants could synthesize both RMF and the s38 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/...
pUCglpFK or E. coli CAG2242/pUCglpFK 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 1-glycerol 3-phosphate in E. coli CAG2242 and the revertant SR-199 were measured. As shown in Table 1, levels of 1-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 1-glycerol 3-phosphate also increased when E. coli CAG2242 was transformed with pUCglpF, pUCglpK, and pUCglpFK (Table 1). It was maximal with E. coli CAG2242/pUCglpFK, indicating that the level of 1-glycerol 3-phosphate correlates with the degree of recovery of cell viability. Addition of 15 mM 1-glycerol 3-phosphate also restored cell viability significantly (data not shown).

### Table 1. Levels of 1-glycerol 3-phosphate in various E. coli strains

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Culture time (h)</th>
<th>1-Glycerol 3-phosphate conc (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG2242</td>
<td>12</td>
<td>2.74 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.81 ± 0.14</td>
</tr>
<tr>
<td>SR-199</td>
<td>12</td>
<td>6.92 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7.31 ± 0.18</td>
</tr>
<tr>
<td>CAG2242/pUC118</td>
<td>24</td>
<td>1.80 ± 0.18</td>
</tr>
<tr>
<td>CAG2242/pUCglpFK</td>
<td>24</td>
<td>4.52 ± 0.11</td>
</tr>
<tr>
<td>CAG2242/pUCglpFK</td>
<td>24</td>
<td>4.61 ± 0.13</td>
</tr>
<tr>
<td>CAG2242/pUCglpFK</td>
<td>24</td>
<td>5.81 ± 0.14</td>
</tr>
</tbody>
</table>

* Cells were cultured in the absence of spermidine.

#### DISCUSSION

The results of this study show that 1-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 1-glycerol 3-phosphate, directly interact with polyamines and thereby modulate their functions. We also reported recently that the
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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